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**Endothelial Injury and Repair in Childhood  
Arterial Ischaemic Stroke**

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I, Despina Eleftheriou confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in this thesis.

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## Thesis abstract

Abnormalities of the cervical or intracranial circulation, termed arteriopathies are the leading mechanism of both cause and recurrence of childhood arterial ischaemic stroke (AIS). Approximately 20% of children with AIS will have stroke recurrence but there are currently no robust biomarkers to identify this high risk group, and hence identification of patients who may be amenable to secondary preventative strategies has not been possible. This thesis attempts to address this unmet need by studying novel biomarkers to distinguish patients at risk of stroke recurrence. Indices of endothelial injury, repair and hypercoagulability were compared between patients with recurrent clinical disease course and children with a single event. Circulating endothelial cells (CECs) were higher in children with recurrent AIS, compared to those with no recurrence and controls. Further evidence of endothelial injury and cellular activation was derived by examining circulating microparticles (MP) profiles. Plasma from patients with AIS recurrence contained increased numbers of endothelial, platelet and neutrophil derived MP compared to those with no recurrence. These MPs were highly prothrombotic due to phosphatidylserine exposure providing a platform for the assembly and activation of coagulation factors; and also expression of tissue factor on some MP. An efficient in vitro assay to assess MP-related hypercoagulability by quantifying MP-mediated thrombin generation was established. Children with recurrent AIS were shown to have an enhanced MP-mediated thrombin generation. Lastly, a disturbance in endothelial progenitor cells (EPCs) in children with AIS recurrence was observed suggesting that there could be impairment of endothelial repair in these patients. In conclusion, despite the wide spectrum of clinical and radiological presentation of childhood AIS, the studies undertaken in this thesis suggest that there is an unfavourable imbalance between endothelial injury and

repair, and excess hypocoagulability in children with recurrent AIS. These novel observations provide unique insights into the pathophysiology of paediatric AIS.

## Abbreviations

Abbreviation	Definition
ACR	American College of Rheumatology
AIS	Arterial ischaemic stroke
AUC	Area under the curve
BSA	Bovine serum albumin
BVAS	Birmingham Vasculitis Activity Score
CA	Catheter angiography
CAA	Coronary artery abnormality
cANCA	Cytoplasmic antinuclear cytoplasmic antibody
CEC	Circulating endothelial cells
CNS	Central nervous system
CRP	C reactive protein
CSF	Cerebro-spinal fluid
CSS	Churg Strauss Syndrome
CT	Computed tomography
EC	Endothelial cell
ELISA	Enzyme linked immunosorbant assay
EMP	Endothelial microparticle
EPC	Endothelial progenitor cells
ESR	Erythrocyte sedimentation rate
ETP	Endogenous thrombin potential
FACS	Flow activated cell sorter
FCS	Foetal calf serum
GPA	Granulomatosis with polyangiitis
HMEC	Human microvascular endothelial cell
HSP	Henoch Schönlein Purpura
HUVEC	Human umbilical endothelial cell
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
INF- $\gamma$	Interferon- $\gamma$
IVIG	Intravenous immunoglobulin
KD	Kawasaki disease
LR	Likelihood ratio
MFI	Median fluorescence index
MP	Microparticle
MMP	Monocyte microparticle
MPO	Myeloperoxidase
MRI	Magnetic resonance imaging
MRA	Magnetic resonance angiography
MS	Multiple sclerosis
NMP	Neutrophil microparticles
NO	Nitric oxide
NPV	Negative predictive value

PAN	Polyarteritis nodosa
pANCA	Perinuclear anti neutrophil cytoplasmic antibody
PBMC	Peripheral blood mononuclear cells
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PMP	Platelet microparticle
PPV	Positive predictive value
PR3	Proteinase 3
PS	Phosphatidylserine
PSGL-1	P selectin glycoprotein ligand-1
ROC curve	Receiver operator characteristic curve
SCD	Sickle cell disease
SEM	Standard error of the mean
SLE	Systemic lupus erythematosus
SM	Sphingomyelin
SV	Systemic vasculitis
TA	Takayasu arteritis
TGA	Thrombin generation assay
TNF- $\alpha$	Tumour necrosis factor- $\alpha$
TPA	Tissue plasminogen activator
VCAM-1	Vascular cell adhesion molecule-1
VZV	Varicella zoster virus
WG	Wegener's granulomatosis
WHO	World Health Organisation

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# **1 Introduction**

## **1.1 Arterial ischaemic stroke in children**

### ***1.1.1 Definitions***

Stroke is defined by the World Health Organisation as “a clinical syndrome typified by rapidly developing signs of focal or global disturbance of cerebral functions, lasting more than 24 hours or leading to death, with no apparent causes other than of vascular origin”(Mackay et al. 2004).

The broad definition of paediatric stroke includes ischaemic and haemorrhagic stroke (Amlie-Lefond et al. 2008). Ischaemic stroke (IS), includes arterial ischaemic stroke (AIS) and cerebral sinovenous thrombosis (CSVT), whereas haemorrhagic stroke includes intra-cerebral and subarachnoid haemorrhage (Amlie-Lefond et al. 2008). AIS is defined as an acute-onset focal neurological deficit attributable to cerebral infarction in an arterial distribution associated with this clinical presentation (Mackay et al. 2011).

Paediatric stroke can also be subdivided according to age of onset into ischaemic perinatal stroke (defined as a group of heterogeneous conditions in which there is focal disruption of cerebral blood flow secondary to arterial or cerebral venous thrombosis or embolization, between 20 weeks of fetal life through the 28th postnatal day, confirmed by neuroimaging or

neuropathologic studies) and later childhood stroke (Amlie-Lefond et al. 2008; Raju et al. 2007). This thesis will focus on later childhood AIS as defined above.

### ***1.1.2 Epidemiology***

Worldwide, stroke is the third most common cause of death, responsible for over 5 million deaths per year (Gustavsson et al. 2011; Hachinski et al. 2010). Many of these patients are left with permanent neurologic deficits, and stroke incurs a high cost to patients and their families, health care providers and societies (Gordon et al. 2002; MacGregor et al. 2000). The total cost of disorders of the brain in Europe was estimated at €798 billion in year 2010, with stroke accounting for 8% of these costs (Gustavsson et al. 2011). In the light of this, remarkable efforts have been made over the last 10 years to increase public recognition of adult stroke, develop prevention strategies and improve treatment (Hachinski et al. 2010). In contrast childhood stroke is seldom considered as a significant health care problem (Amlie-Lefond et al. 2008; deVeber 2003). However among childhood diseases, stroke is not rare and the burden of illness related to the neurological damage and mortality from this disorder is significant (Fullerton et al. 2002). Stroke in children is at least as frequent as brain tumours and is among the top ten causes of death in childhood (Fullerton et al. 2002). In the US the cost per paediatric stroke acute admission ranges from \$20 000 to \$81 000 with 5-year costs of \$135 000 (Perkins et al. 2009). Health care utilisation costs and loss of productive years when stroke results in significant neurological or psychological deficits are also significant contributors to the burden of illness (Gardner et al. 2010; Lo et al. 2008). In addition it is likely that the cost of recurrent strokes over the lifetime of children with the

disease may be considerably increased compared to adults (Gardner et al. 2010; Lo et al. 2008).

Over the last 30 years several population-based studies have reported on varying incidence of childhood stroke (deveber 2000; Fullerton et al. 2003; Mallick and O'Callaghan 2010). Among these studies the inclusion criteria, methods of patient identification and geographical population evaluated have differed, with each study contributing differently to our understanding of incidence and subtypes of stroke (deveber 2000; Fullerton, Wu, Zhao, & Johnston 2003; Mallick & O'Callaghan 2010). Data from the Canadian Paediatric Ischaemic Stroke Registry provide an estimated incidence of 3.3 per 100,000 children per year for childhood ischaemic stroke (AIS and cerebral venous thrombosis) (deveber 2000). Retrospective analysis of a California-wide hospital discharge database estimated an incidence of stroke (AIS, cerebral venous thrombosis, and haemorrhagic stroke including subarachnoid haemorrhage) of 2.3 per 100,000 children per year (Fullerton, Wu, Zhao, & Johnston 2003). In addition a prospective study across United Kingdom and Ireland reported an incidence of stroke (AIS, cerebral venous thrombosis, and haemorrhagic stroke including subarachnoid haemorrhage) of 2.5 per 100,000 children (Williams et al. 2002). It is likely however that these figures are an underestimate, as the index of suspicion for AIS is typically low in the paediatric population.

The risk of childhood stroke considerably varies according to age (Barnes et al. 2004; Fullerton, Wu, Zhao, & Johnston 2003; Ganesan et al. 2003). The majority of the studies report the greatest risk of childhood stroke in children aged less than 1 year (Barnes et al.

2004; Fullerton, Wu, Zhao, & Johnston 2003). In studies that analysed neonates (aged less than 1 month) separately, this group was found to be at particularly high risk, responsible for up to 35% of cases of childhood stroke (Barnes et al. 2004; Steinlin et al. 2005). Both Fullerton et al. and Kirkham et al. reported that after the age of 1 year the risk of overall stroke fell significantly between the ages of 5 and 9 years before rising again in adolescence (Fullerton, Wu, Zhao, & Johnston 2003; Kirkham et al. 2003).

In addition, several paediatric studies have shown a male predominance in children with AIS (Golomb et al. 2009; Mackay et al. 2011). The International Paediatric Stroke Study Group (IPSS) found a male to female ratio of 1.49 amongst the first 1187 patients enrolled (Mackay et al. 2011). A number of factors have been postulated to explain the male gender predominance. Hormonal factors such as oestrogen that has vaso-dilatory and anti-inflammatory effects may offer relative protection from stroke to females (Mallick & O'Callaghan 2010; Reeves et al. 2008). Among boys, elevated testosterone levels have been shown to be independently associated with stroke risk (Golomb, Fullerton, & Nowak-Gottl 2009). Differences in sports and other physical activities that have been associated with certain types of childhood stroke such as arterial dissection and AIS may partially explain gender differences too (Fullerton et al. 2001; Mallick & O'Callaghan 2010). Of note in the Californian cohort, the risk of stroke for black children was twice that for white children while the relative risk for Hispanics compared to whites was 0.76 and Asians and whites had equal risk (Fullerton, Wu, Zhao, & Johnston 2003). This increased risk remained significant even when cases of sickle cell disease were excluded (Fullerton, Wu, Zhao, & Johnston 2003).

### ***1.1.3 Clinical Presentation***

Childhood AIS commonly presents with acute hemiparesis (Ganesan and Kirkham 2011). Patients whose signs resolve within 24 hours or longer have transient ischaemic attacks (TIA) but may have cerebral infarction on imaging (Ganesan et al. 2003; Ganesan & Kirkham 2011). Other presenting features include altered mental status, aphasia and visual disturbance (Fox et al. 2012; Ganesan & Kirkham 2011). Children may also present with signs of raised intracranial hypertension and imminent central or uncal herniation and may require admission to paediatric intensive care units (Fox, Johnston, Sidney, & Fullerton 2012; Ganesan & Kirkham 2011). Seizures and status epilepticus have also been described and are associated with a high likelihood of developing epilepsy later on in life (Chadhumbe et al. 2009). In addition, coma is a well recognised presentation in children with large middle cerebral artery (MCA) territory infarct (Fox, Johnston, Sidney, & Fullerton 2012; Ganesan & Kirkham 2011). Stroke in the posterior circulation can present as ataxia, vertigo, or vomiting (Ganesan et al. 2002). Younger children are more likely to present with encephalopathy and a decreased level of consciousness; and may on detailed neurological examination be found to have a focal neurological deficit (Zimmer et al. 2007). The rapidity of onset may help decide which investigations should be undertaken as abrupt onset (< 30 minutes to complete deficit) was documented in more than two thirds of those subsequently found to have non-arteriopathic AIS for example embolism, compared with only 32% of arteriopathic stroke (Braun et al. 2007).

The differential diagnosis of children presenting with acute focal neurological deficit is wide and in addition to stroke includes: encephalitis, brain tumors, hemiplegic migraine, unilateral



hemispheric cerebral oedema (e.g secondary to diabetes, hyperammonaemia), haematoma, posterior reversible encephalopathy syndrome, venous sinus thrombosis, demyelinating disorders and mitochondrial encephalopathy with stroke-like episodes (Ganesan & Kirkham 2011; Kirkham 1999; Shellhaas et al. 2006). Lack of clinical suspicion and the high frequency of these stroke mimics in the young, are some of the probable reasons for common delay in childhood stroke recognition (Braun et al. 2006; McGlennan and Ganesan 2008; Rafay et al. 2009).

#### ***1.1.4 Imaging confirmation of childhood arterial ischaemic stroke (AIS)***

Magnetic resonance imaging (MRI) is the imaging modality of choice for the investigation of paediatric AIS for the confirmation of stroke and the exclusion of alternative treatable pathologies (Fullerton et al. 2007; Jones, Ganesan, Saunders, & Chong 2010). The characteristic radiological features of AIS seen in children are similar to those of young adults (Hunter 2002; Jones, Ganesan, Saunders, & Chong 2010). MR imaging usually includes standard T2-weighted axial images that show tissue contrast well and sagittal and coronal T1 weighted images that maximize contrast between the cerebral tissue and cerebrospinal fluid (CSF) and provide morphological information (Baird and Warach 1998; Hunter 2002; Jones, Ganesan, Saunders, & Chong 2010). In addition fluid-attenuated inversion recovery (FLAIR) suppression of the signal from the CSF increases the visibility of periventricular lesions (Hunter 2002; Jones, Ganesan, Saunders, & Chong 2010). Of note, diffusion weighted imaging (DWI) is able to detect ischaemic regions of the brain before any changes are detectable on conventional MRI (Gadian et al. 2000; Jones, Ganesan, Saunders,

& Chong 2010). The biophysical mechanisms underlying these diffusion changes may be associated with cerebral oedema (Jones, Ganesan, Saunders, & Chong 2010). In addition, perfusion imaging allows demonstration of blood flow within the arterioles and capillaries of the brain (Calamante et al. 1999; Gadian et al. 2000; Jones, Ganesan, Saunders, & Chong 2010).

Immediate access to an MRI unit able to provide a timely and accurate diagnosis should be the gold standard approach (Jones, Ganesan, Saunders, & Chong 2010). However, practical considerations apply with regard to limitations of access to suitable imaging facilities (Jones, Ganesan, Saunders, & Chong 2010). Computed tomography (CT) scanning is still performed in some centres as it is more widely available and still has a role in excluding stroke mimics such as intracerebral haemorrhage (Jones, Ganesan, Saunders, & Chong 2010). Acute infarcts however are more visible on MRI than CT, with over 80% of MRIs positive within the first 24 hours compared with only 60% visible on CT (Bryan et al. 1991; Jones, Ganesan, Saunders, & Chong 2010). MRI is particularly superior in the detection of infarcts within the posterior circulation where CT is limited by beam hardening artifact from the skull base (Bryan et al. 1991; Jones, Ganesan, Saunders, & Chong 2010). In addition lacunar infarcts and small infarcts are better visualised with MRI (Wardlaw et al. 2001).

Magnetic resonance spectroscopy (MRS) is a non-invasive method that allows the in vivo investigation of biochemical changes in stroke (Barker et al. 1994; Gillard et al. 1996). The application of this technique has made possible dynamic studies of intracellular metabolism

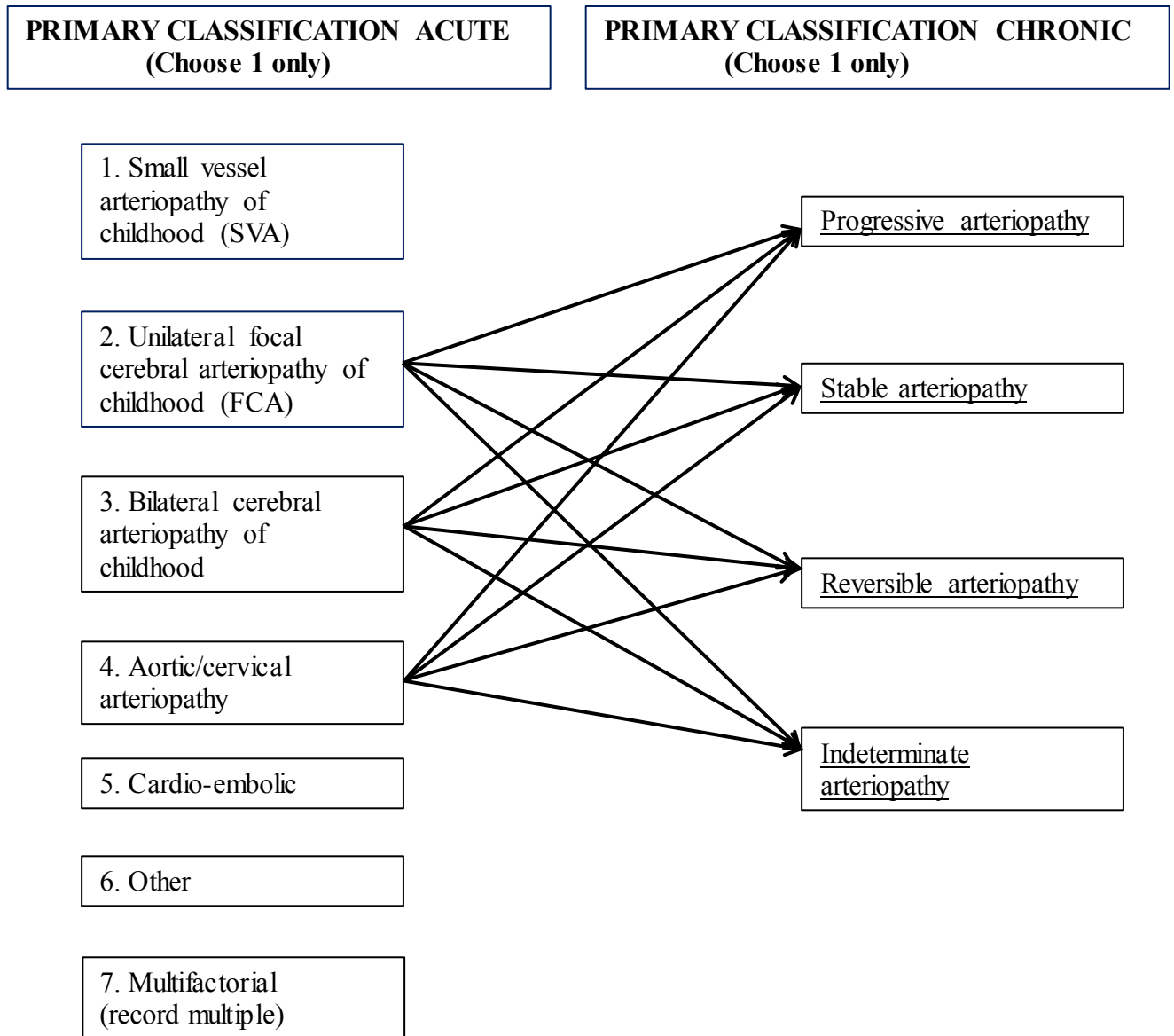
in cerebral ischaemia but to date remains a research tool (Barker et al. 1994; Gillard et al. 1996).

As far as infarct location is concerned, the majority of strokes in childhood AIS occur within the anterior circulation commonly involving the MCA territories (Ganesan et al. 1999; Jones, Ganesan, Saunders, & Chong 2010). The posterior circulation appears to be involved more frequently in cranio-cervical arterial dissection (Ganesan et al. 2002; Mackay et al. 2010). Mackay et al. have recently reported on a cohort of children with posterior circulation AIS (Mackay, Prabhu, & Coleman 2010). Stroke subtypes included dissection, nonprogressive steno-occlusive cerebral arteriopathy, cardioembolic, moyamoya syndrome and other undetermined aetiology (Mackay, Prabhu, & Coleman 2010). Lastly, lacunar infarcts are rare in children (Williams et al. 1997).

### ***1.1.5 Classification of Childhood Arterial Ischaemic Stroke (AIS)***

The adoption of a uniformly agreed nomenclature and the subsequent validation of a classification system in adult AIS have been critical to the study of neurological outcomes, risk-stratified therapies and estimation of recurrence risk (Kang et al. 2003; Kolominsky-Rabas et al. 2001). Over the past ten years, there was considerable progress in understanding the epidemiology, pathophysiology, outcomes, and prognostic factors for recurrence in childhood AIS (Amlie-Lefond et al. 2009; Bernard et al. 2012; Ganesan et al. 2003; Golomb, Fullerton, & Nowak-Gottl 2009; Mackay et al. 2011). However current research efforts are hampered by the lack of standardized nomenclature and classification systems to allow

distinction of various patient groups, enable comparisons for prospective studies of natural history and facilitate interventional therapeutic clinical trials as in adults (Bernard et al. 2012). Use of adult criteria is not feasible as the majority of children with AIS do not meet criteria for atherothrombotic disease (Bernard et al. 2012). Efforts for establishing childhood-specific definitions and classification in AIS have been proposed over the years (Wraige et al. 2005). For instance, Wraige et al. in 2005 proposed a classification system of AIS identifying eight aetiological subtypes: sickle cell disease, cardioembolic, moyamoya syndrome, cervical arterial dissection, steno-occlusive cerebral arteriopathy, other determined aetiology, other undefined aetiology and cases with multiple aetiologies (Wraige et al. 2005). In addition, Sebire et al. proposed definitions for childhood arteriopathies (Sebire et al. 2004). To date however no single system has been widely adopted. With that objective in mind the IPSS established a working group in childhood AIS classification in November 2007 that subsequently developed the Childhood AIS Standardized Classification and Diagnostic Evaluation (CASCADE) criteria (Bernard et al. 2012). Since the pathophysiology of childhood AIS is established in some cases, but not others, the primary classification in this system is based upon the anatomic location of any noted anomaly from the heart to the cerebrum (figure 1-1) (Bernard et al. 2012). The greatest subdivision occurs within the arteriopathies with an initial vascular diagnosis and a follow-up diagnosis at 3-6 months that aims to capture any progression, stability or regression of the arteriopathy (primary classification chronic; figure 1-1) (Bernard et al. 2012). The CASCADE criteria are described in detail in appendix 1. This classification system for childhood AIS has been validated and is expected to be widely adapted in future studies.



**Figure 1-1: CASCADE Criteria Acute Primary Classification: anatomic features (adapted from Bernard et al. 2012).**

CASCADE= Childhood AIS Standardized Classification and Diagnostic Evaluation;  
 AIS=Arterial ischaemic stroke.

## **1.2 Risk Factors for childhood arterial ischaemic stroke (AIS)**

### ***1.2.1 Introduction***

Overall risk factors for AIS differ in children compared with adults (deveber 2003; Fullerton, Wu, Sidney, & Johnston 2007; Ganesan et al. 2003; Mackay et al. 2011). Adult risk factors for stroke relating to atherosclerosis (eg. hypertension, hyper-lipidaemia, diabetes, and smoking) are rarely thought to be related to AIS in children (deveber 2003; Fullerton, Wu, Sidney, & Johnston 2007; Ganesan et al. 2003; Mackay et al. 2011). Also in contrast to adults, socioeconomic status has not been shown to be associated with an increased stroke risk in children (deveber 2003; Fullerton, Wu, Sidney, & Johnston 2007; Ganesan et al. 2003; Mackay et al. 2011). Several studies of childhood AIS have reported on a wide range of underlying systemic risk factors in the setting of childhood stroke, particularly sickle cell disease, cardiac disorders, trauma, thrombophilia and infections such as meningitis, sepsis, and encephalitis (Mackey et al 2011; deveber 2003; Fullerton, Wu, Sidney, & Johnston 2007; Ganesan et al. 2003). Lo et al. identified congenital heart disease and infections (sepsis and meningitis/encephalitis) as commonly associated with childhood AIS in a US cohort ( Lo et al. 2009). Of note arrhythmias, hypertension, and coagulation defects were associated with both types of ischaemic and haemorrhagic stroke in that study (Lo et al. 2009). Ganesan et al. reported data on the known risk factors encountered in children presenting with a first AIS in a single UK based center (Ganesan et al. 2003). Cerebral arterial imaging was abnormal in 79% of patients who had vascular imaging, providing evidence that cerebral arteriopathies are important risk factors associated with AIS (Ganesan et al. 2003). Eight out of the 104 patients investigated with echocardiography, had

abnormal studies (Ganesan et al. 2003). Genetic or acquired conditions causing thrombophilia were rare in that study (Ganesan et al. 2003). Forty percent of patients were anaemic, and 21% either had elevated total plasma homocysteine or were homozygous for the thermolabile variant of the methylene tetrahydrofolate reductase gene (t-MTHFR) mutation (Ganesan et al. 2003). In addition, the most recent study by the IPSS, reported on a large group of children (a total of 676 children) with AIS across a number of geographical regions (Mackay et al. 2011). Risk factors associated with AIS included arteriopathies (53%), cardiac disorders (31%), infection (24%), acute head and neck disorders (23%), acute systemic conditions such as sepsis and dehydration (22%), chronic systemic conditions such as sickle cell disease and connective tissue disorders (19%), prothrombotic states (13%), chronic head and neck disorders (10%), atherosclerosis-related risk factors such as hypertension and hyperlipidaemia (2%), and other (22%) (Mackay et al. 2011). Importantly 52% of the patients studied had more than 2 risk factors identified (Mackay et al. 2011). Risk factors in this study varied by age group; arteriopathy was most common in children aged 5 to 9 years, acute systemic conditions were more common in younger children, and chronic head and neck disorder in older children (Mackay et al. 2011). The study also reported on a lower prevalence of arteriopathy in Asia, lower prevalence of chronic systemic conditions in Europe and Australia, higher prevalence of prothrombotic states in Europe, and higher prevalence of acute systemic conditions in Asia and South America (Mackay et al. 2011). Of note, variation in the diagnostic approach of these patients may contribute to the reported differences. Vascular imaging for instance was less frequently undertaken in Asia and South America than in other parts of the world, possibly reflecting the local healthcare setting or resource limitations and resulting in reduced frequency of detection of arteriopathy

(Mackay et al. 2011). Not surprisingly, the prevalence of acute systemic illnesses was relatively higher in Asia and South America (Mackay et al. 2011).

In summary, several studies confirm that risk factors, commonly multiple, are usually identified in childhood AIS; these appear to vary according to age and geography (Lo et al. 2009; Ganesan et al. 2003; Mackay et al. 2011). The most important risk factors associated with childhood AIS appear to be nonatherosclerotic arteriopathies and cardiac disease, and this is the case across several geographical regions (Lo et al. 2009; Ganesan et al. 2003; Mackay et al. 2011). The following sections summarise our current knowledge for all established risk factors implicated in the pathogenesis of childhood AIS. The presence and morphology of arteriopathy as the single most important determinant of acute management and of recurrence risk are highlighted.

### ***1.2.2 Cerebral arteriopathy***

Cerebral arteriopathy, defined as arterial disease identified on vascular imaging, is one of the most common findings in the diagnostic evaluation of children presenting with AIS (Braun et al. 2009; Ganesan et al. 2003; Mackay et al. 2011). A California based study reported that of 52 children who had vascular imaging after AIS, 42% had stenosis due to arterial lesions (Fullerton, Wu, Sidney, & Johnston 2007). A larger study in the UK, reporting on a hospital series of 185 children with AIS, found vascular abnormalities in 147 (79%); the most common intracranial abnormalities were occlusion or narrowing of the proximal large arteries, which was seen in 95 patients (64%) (Ganesan et al. 2003). The recent IPSS also



reported that the majority of children who underwent cerebrovascular imaging had evidence of cerebral arterial abnormalities (Mackay et al. 2011).

Sebire et al. have summarised the spectrum of arteriopathies encountered in children with AIS with varying frequency, including moyamoya, vasculitis, dissection, transient cerebral arteriopathy (TCA) and post varicella arteriopathy (PVA) (table 1-1) (Sebire et al. 2004).

The nomenclature and classification for these arteriopathy subtypes are still in evolution.

Non-inflammatory vasculopathies

Transient cerebral arteriopathy (TCA)

Arterial dissection

Sickle cell disease arteriopathy

Moyamoya syndrome and primary moyamoya disease

Neurofibromatosis

Tuberous sclerosis

Downs syndrome

Post irradiation

Congenital vessel hypoplasia/dysplasia

Fibromuscular dysplasia

Vascular webs

Drug-related vasculopathy

Cocaine, Amphetamine, Methylphenidate, Phenylpropanolamine

Primary vasculitides with CNS involvement

Large vessel

-Takayasu arteritis

Medium vessel

-Polyarteritis nodosa

-Kawasaki disease

Small vessel

-Primary CNS angitis

-Churg-Strauss syndrome,

-Wegener's granulomatosis

-Henoch-Schönlein purpura

Secondary vasculitides with CNS involvement

Collagen vascular diseases

Systemic lupus erythematosus

Mixed connective tissue disease

Infectious vasculitis

Viral

-Varicella zoster

-Cytomegalovirus

-HIV

Bacterial

-Septic meningitis

-Lyme disease

-Tuberculosis

Mycoses

-Coccidioides

-Aspergillus

Parasites

-Cysticercus

**Table 1-1: Cerebral arteriopathies associated with childhood arterial ischaemic stroke (AIS). Adapted from Sebire et al.2004**

### **1.2.2.1 Focal cerebral arteriopathy (FCA)/ transient cerebral arteriopathy (TCA)/Post varicella arteriopathy (PVA)**

Historically, occlusive arteriopathies with no features of dissection or moyamoya were described as idiopathic arteriopathies (Chabrier et al. 1998; Sebire 2006). Unilateral cases were further classified retrospectively as transient cerebral arteriopathy (when improving over time) or chronic cerebral arteriopathy (if persistent) (Chabrier et al. 1998; Sebire 2006). The diagnostic criteria for transient cerebral arteriopathy (TCA) are that initial vascular imaging (within 3 months of the infarct) should show unilateral or focal segmental stenosis or occlusion involving the distal part of the internal carotid and initial segments and branches of the anterior and/or middle cerebral artery, though initial imaging can sometimes only find minimal or no stenosis or occlusion that then increases to a maximum stenosis or occlusion within 3 months of the infarct (Chabrier et al. 2006). The second criterion is that there should be non-progression or regression of the arterial lesions on follow-up imaging 6 months after the infarct (Chabrier et al. 1998; Sebire 2006). It should be noted that 6 months is an arbitrary time period and it may be that cases which show improvement after considerably longer periods (up to 93 months in one study) may share the same natural history as those with improvement in the arteriopathy by 6 months (Chabrier et al. 1998; Sebire 2006). In some sense, the term “transient” cerebral arteriopathy is somewhat misleading. Although monophasic in the progression of stenosis, in many cases the arterial narrowing persists on long-term follow-up imaging. Furthermore when similar angiographic appearances are preceded by VZV infection in the preceding 12 months the arteriopathy is termed post varicella arteriopathy (PVA) (Lanthier et al. 2005; Sebire et al. 2004). Lanthier

et al. reported on the course of vascular changes in childhood PVA and its relationship to recurrent AIS or transient ischaemic attacks (TIAs) (Lanthier, Armstrong, & Domi 2005). This study suggested that vascular stenosis of childhood PVA takes a monophasic course, generally with subsequent stenosis regression and only occasional stenosis progression after AIS/TIA (Lanthier, Armstrong, & Domi 2005). In addition, Miravet et al. recently reported on 24 children with AIS at median of 4 months following VZV infection (Miravet et al. 2007). All had infarction in the middle cerebral artery (MCA) territory and abnormalities of the M1 segment with arteriopathy affecting other arteries in 10 children (Miravet et al. 2007). Arterial disease improved in 11 children, was stable in four, and progressed in seven (of whom four had recurrent TIA and two had re-infarction) (Miravet et al. 2007). In general, this terminology is somewhat confusing as it encompasses description of disease processes (TCA) and aetiology (PVA) as well as heavily relying on radiological appearances to distinguish between these arteriopathies.

In recognition of the limitations of classifying arteriopathies on initial imaging, the IPSS has recently suggested the term “focal cerebral arteriopathy” of childhood (FCA) to describe children with focal stenotic disease of large and/or medium sized vessels not meeting definitions for moyamoya, sickle cell vasculopathy, dissection or vasculitis and commonly with no identifiable aetiology (Amlie-Lefond et al. 2009). FCA represents up to 25% of all arteriopathies (Amlie-Lefond et al. 2009). This term is likely to replace TCA and does not depend on the long term course of these arteriopathies (Amlie-Lefond et al. 2009). It must be noted however that both the terms TCA and FCA are simply descriptive and do not imply a specific pathophysiology. PVA is considered a subset of FCA characterised by unilateral

stenosis and AIS within 1 year of varicella infection. The typical course of FCA is an initial worsening of the cerebral arterial stenosis within the first 3 to 6 months, followed by stabilization or improvement (Amlie-Lefond et al. 2009). Braun et al. characterized this pattern of vascular changes in 94% of 79 children with unilateral intracranial arteriopathy and AIS included in their study, while 6% in their cohort progressed to moyamoya (Braun et al. 2009). Of the cases with non-moyamoya AIS, complete resolution occurred in 24%, improvement without resolution occurred in 45%, and stabilization occurred in 32% (Braun et al. 2009).

#### **1.2.2.2 Arterial dissection**

Intracranial or cervical arterial dissection is increasingly recognised in association with AIS (up to 20% cases in some series), particularly in adolescents and is the only vascular diagnosis where acute anticoagulation is generally recommended (Rafay et al. 2006; Roach et al. 2008; Royal College of Physicians, 2004). A traumatic injury is the most frequent cause of a dissection; this includes major or minor direct neck or head trauma. However some cases are attributed to connective tissue disorders such as Ehlers-Danlos or Marfan syndrome while an immediate cause for dissection is often unclear (North et al. 1995; Yetman et al. 2003). Confirmation of the diagnosis of intracranial or cervical arterial dissection requires MRI/MRA or catheter angiography with one of the following three patterns: (1) angiographic findings of a double lumen, intimal flap, or pseudo aneurysm, or, on axial T1 fat saturation MRI images, a “bright crescent sign” in the arterial wall; (2) the sequence of cervical or cranial trauma, or neck pain, less than 6 weeks preceding angiographic findings of segmental arterial narrowing (or occlusion) located in the cervical

arteries; (3) angiographic segmental narrowing (or occlusion) of the vertebral artery at the level of the C2 vertebral body, even without known traumatic history (Sebire et al. 2004).

### **1.2.2.3 Primary angiitis of the central nervous system**

Central nervous system (CNS) vasculitis in children is an increasingly recognized inflammatory brain disease that may occur as a primary disease isolated to the CNS (primary angiitis of the CNS, PACNS) or as a secondary manifestation of an underlying systemic condition (Benseler et al. 2006; Calabrese and Mallek 1988). A number of systemic inflammatory diseases (systemic lupus erythematosus, primary systemic vasculitides, Behçets disease, scleroderma amongst others) and infections (streptococcus, mycoplasma pneumonia, mycobacterium tuberculosis, Epstein Barr virus, enteroviruses, human immunodeficiency virus, cytomegalovirus, candida, aspergillus amongst many others) can cause secondary CNS vasculitis (Benseler et al. 2006; Cellucci and Benseler 2010; Elbers and Benseler 2008).

Diagnostic criteria for PACNS were proposed by Calabrese et al. in 1992 and were subsequently adapted for childhood PACNS (cPACNS) (Calabrese & Mallek 1988; Elbers & Benseler 2008). These include the following:

1. The presence of an acquired otherwise unexplained neurological or psychiatric deficit.
2. The presence of either classic angiographic or histopathological features of angiitis within the CNS.

3. No evidence of systemic vasculitis or any disorder that could cause or mimic the angiographic or pathological features of the disease.

Patients should meet all three criteria to be diagnosed with PACNS (Calabrese & Mallek 1988). Childhood PACNS (cPACNS) mandates a patient age of  $\leq 18$  years at diagnosis and excludes neonates (1 month of age) (Cellucci & Benseler 2010). Of note these diagnostic criteria even though widely used have not been validated (Cellucci & Benseler 2010; Elbers et al. 2010; Hutchinson et al. 2010). Childhood PACNS is broadly subdivided into two forms of the disease: (i) angiography positive large–medium vessel vasculitis (further divided into progressive and non-progressive, according to angiographic evidence of disease progression 3 months after diagnosis); (ii) angiography negative small vessel vasculitis confirmed by brain biopsy (Cellucci & Benseler 2010; Elbers et al. 2010).

The clinical presentation of cPACNS is heterogeneous, with some children presenting with a rapidly progressive neurologic deficit, whereas others have a slowly evolving disease course over weeks or months (Benseler et al. 2006; Hutchinson et al. 2010). Headache is a common symptom and tends to vary in description, intensity, and pattern (Benseler et al. 2006; Hutchinson et al. 2010). Cognitive impairment can be insidious while stroke and TIAs are common (Benseler et al. 2006; Hutchinson et al. 2010). Additionally cranial nerve involvement including ocular nerves in addition to myelopathy, seizures and ataxia have been described (Benseler et al. 2006; Hutchinson et al. 2010). Children with angiography positive large to medium vessel cPACNS are likely to present with focal neurologic deficits, including hemiparesis, hemisensory deficits, fine motor deficits, and dysphagia while diffuse neurological deficits, such as cognitive dysfunction and behaviour changes are more

common in the group of children with small vessel vasculitis (Benseler et al. 2006; Cellucci et al. 2011). Seizures and constitutional symptoms are less common (Benseler et al. 2006; Cellucci, Tyrrell, Sheikh, & Benseler 2011; Hutchinson et al. 2010).

A wide range of inflammatory and non-inflammatory disorders can mimic CNS vasculitis, summarized in table 1-2 and need to be considered in the differential diagnosis (Cellucci & Benseler 2010; Elbers & Benseler 2008). In terms of establishing a diagnosis of cPACNS there are no consistent or reliable laboratory abnormalities and normal inflammatory markers by no means exclude an active vasculitic process in the CNS (Cellucci & Benseler 2010; Elbers & Benseler 2008). Conventional acute phase reactants including erythrocyte sedimentation rate, and concentrations of C-reactive protein, C3 complement maybe raised while von-Willebrand-factor antigen is commonly mildly increased (Cellucci et al. 2009; Cellucci et al. 2012; Cellucci & Benseler 2010; Elbers & Benseler 2008). Cerebrospinal fluid (CSF) analysis might show a raised opening pressure, mild pleocytosis with predominant lymphocytes, and mildly raised protein concentration (Cellucci & Benseler 2010; Elbers & Benseler 2008; Hutchinson et al. 2010). MRI brain/spine in cPACNS reveals areas of acute ischemia in a vascular distribution when large-medium vessels are affected. In cases of small vessel disease, the lesions may be multifocal and not necessarily conform to a specific vascular distribution (Cellucci & Benseler 2010; Elbers & Benseler 2008). The parenchymal lesions may involve both grey and white matter, and meningeal enhancement has also been described (Cellucci & Benseler 2010; Elbers & Benseler 2008). Diffusion weighted imaging (DWI) identifies areas of ischaemia in large vessel disease (Cellucci & Benseler 2010; Elbers & Benseler 2008). MRA provides an assessment of the vasculature



and may reveal beading, tortuosity, stenosis, and occlusion of the vessels (Aviv et al. 2006; Cellucci & Benseler 2010; Elbers & Benseler 2008). Conventional CA continues to be the radiological gold-standard for identifying cerebrovascular changes in patients with suspected CNS vasculitis and is more sensitive than MRA at detecting distal lesions that affect small caliber vessels, and for lesions in the posterior part of the brain (Aviv et al. 2006; Cellucci & Benseler 2010; Elbers & Benseler 2008).

Brain biopsy for confirmation of vasculitis should be considered in difficult cases, but is rarely performed in children due to the invasiveness of the procedure (Cellucci & Benseler 2010; Elbers & Benseler 2008). It should however be strongly considered in cases of high clinical suspicion but with negative arteriography findings or in cases of poor response to therapy (Elbers et al. 2010). Specimens for brain biopsy should contain leptomeninges, cortical gray matter, and subcortical white matter (Elbers et al. 2010). Biopsy findings characteristically reveal segmental, non-granulomatous, intramural infiltration of arteries, arterioles, capillaries, or venules (Elbers et al. 2010). Non lesional biopsy may be considered when lesions identified on imaging are not easily accessible (Elbers et al. 2010). Elbers et al. have recently reported on the clinical and histopathological features of brain biopsies in 13 children with small-vessel cPACNS (Elbers et al. 2010). Findings included intramural lymphocytic infiltrate with reactive endothelial cells and tubular reticular inclusion bodies identified on electron microscopy in 38% of the cases (Elbers et al. 2010). Reticulin staining (performed in some cases), revealed vessel wall reactivity in a lacelike pattern in all cases (Elbers et al. 2010). In adults with small-vessel PACNS studies describing biopsy findings have predominantly shown granulomatous inflammation with multinucleated giant cells

(Elbers et al. 2010). Of note, the intramural distribution of inflammatory cells differentiates PACNS from the perivascular inflammation seen in other T-cell-mediated inflammatory brain diseases (Elbers et al. 2010). Conditions such as Rasmussen encephalitis can be further characterized by microglial nodules and neuronal dropout while in multiple sclerosis there is commonly evidence of myelin-laden macrophages, perivenous or confluent demyelination, and predominant axonal and oligodendrocyte injury (Elbers et al. 2010). The absence of viral inclusions helps differentiate small vessel cPACNS from infectious cerebritis (Elbers et al. 2010).

Celluci et al. recently reported on a series of patients with cPACNS comparing the clinical, laboratory, and imaging characteristics of cPACNS subtypes at diagnosis and during follow-up (Cellucci, Tyrrell, Sheikh, & Benseler 2011). Children with angiography-negative cPACNS in that study were more likely to be female and present with seizures, cognitive dysfunction, vision abnormalities, high inflammatory markers, and bilateral MRI findings; motor deficits and ischaemic MRI lesions were more common in angiography positive disease (Cellucci, Tyrrell, Sheikh, & Benseler 2011). Additionally seizures at diagnosis predicted higher disease activity over time (Cellucci, Tyrrell, Sheikh, & Benseler 2011).

With regards to therapy there are to date no randomised control trials to guide treatment (Cellucci, Pullenayegum, Tyrrell, & Benseler 2009; Cellucci & Benseler 2010; Elbers & Benseler 2008). Current therapeutic recommendations are based on those for systemic vasculitis and include: Six months induction of remission therapy with intravenous cyclophosphamide: 500-1000 mg/m<sup>2</sup> (max 1.2g) every 3-4 weeks (usually 7 doses); plus

corticosteroids and anti-platelet doses of aspirin (Cellucci, Pullenayegum, Tyrrell, & Benseler 2009; Cellucci & Benseler 2010; Elbers & Benseler 2008). Following successful induction of remission there follows 1-2 years maintenance therapy with azathioprine (1.5-3 mg /kg/day), low dose daily or alternate day corticosteroid, and continuation of aspirin (Cellucci, Pullenayegum, Tyrrell, & Benseler 2009; Cellucci & Benseler 2010; Elbers & Benseler 2008). Even less data are available to guide total duration of therapy and the decision to withdraw treatment is individualised per case (Cellucci, Pullenayegum, Tyrrell, & Benseler 2009; Cellucci & Benseler 2010; Elbers & Benseler 2008). Mycophenolate mofetil (MMF) has also been reported to be effective in some cases to maintain remission (Sen et al. 2010). Full anti-coagulation may need to be considered on an individual patient basis (Cellucci, Pullenayegum, Tyrrell, & Benseler 2009; Cellucci & Benseler 2010; Elbers & Benseler 2008). Hutchinson et al. recently reported a single-centre open-label cohort study in 19 children with small vessel cPACNS exploring the efficacy of therapy with steroids and pulses of intravenous cyclophosphamide followed by maintenance therapy with either azathioprine or mycophenolate mofetil (Hutchinson et al. 2010). Thirteen patients completed 24 months' follow-up, nine of whom had a good neurological outcome (Hutchinson et al. 2010). Eight of 19 patients experienced disease flares while 4 patients achieved remission of disease off medication (Hutchinson et al. 2010). Treatment of large vessel non-progressive disease remains controversial (Cellucci & Benseler 2010; Elbers & Benseler 2008). There may be a role for steroids and aspirin without cytotoxic immunosuppression (Cellucci & Benseler 2010; Elbers & Benseler 2008).

As far as the outcome of cPACNS is concerned a recent study of 62 children with cPACNS suggested a poorer prognosis for patients presenting with (1) a neurocognitive dysfunction, (2) multifocal parenchymal lesions on MRI or (3) evidence of distal stenoses on arteriography (Benseler et al. 2006). Further long-term follow-up studies are necessary to accurately define the prognosis of this condition in children (Benseler et al. 2006).

Arterial dissection  
 Thromboembolic disease (congenital heart disease, inherited thrombophilia)  
 Antiphospholipid syndrome  
 Sickle cell disease  
 Moyamoya disease (cerebral arteriopathy characterized by progressive steno-occlusive changes at the terminal portions of the bilateral internal carotid arteries with arterial collateral vessels at the base of the brain)  
 Fibromuscular dysplasia  
 Fabry's disease  
 Sneddon's syndrome (morphologically fixed livedo reticularis and cerebrovascular accidents)  
 CASADIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy)  
 Susac's syndrome (acute encephalopathy, branch retinal artery occlusions and sensorineural hearing loss)  
 Amyloid angiopathy  
 Hyperhomocysteinaemia  
 Drug-exposure (cocaine, amphetamine, methylphenidate)  
 Metabolic diseases  
 Mitochondrial diseases  
 Leukodystrophies  
 Mucopolysaccharidoses  
 Multiple sclerosis  
 Acute disseminated encephalomyelitis (ADEM)  
 Devic's disease/Neuromyelitis optica (CNS demyelinating condition affecting predominantly the spinal cord and optic nerves characterized by the presence of aquaporin-4 water channel IgG antibodies)  
 Vitamin B12 deficiency  
 Rasmussen syndrome (neurological disorder characterized intractable focal seizures, progressive hemiplegia and increasing cognitive impairment)  
 Sarcoidosis  
 Coeliac disease  
 Primary and secondary haemophagocytic lymphohistiocytosis (HLH)  
 Progressive multifocal leukoencephalopathy (JC virus)  
 Lymphoma  
 Glioma  
 Migraine/Vasospasm  
 Post radiation therapy for CNS tumour

***Table 1-2: Mimics of central nervous system (CNS) vasculitis in children.***

Adapted from Elbers et al. 2008.

#### **1.2.2.4 Moyamoya**

First described by Takeuchi and Shimizu, moyamoya is a cerebral arteriopathy with bilateral severe stenosis or occlusion of the terminal internal carotid artery (ICA), typically accompanied by basal collateral vessels (Takeuchi and Shimizu 1957). The name moyamoya derives from the Japanese word meaning ‘something hazy like a puff of smoke drifting in the air’ and describes the angiographic appearance of these collaterals. Moyamoya is best considered an angiographically defined phenomenon rather than a pathological entity and may either be idiopathic or occur in the context of a wide range of other disorders (Natori et al. 1997; Scott and Smith 2009). Most of the patients have infarction after presentation with stroke, TIAs or seizures (Scott & Smith 2009). Calcification is also well documented and haemorrhage can occur even though more common in adults (Kitahara et al. 1986; Mori et al. 2008). The term ‘moyamoya disease’ should be restricted to idiopathic Japanese cases (Fukui et al. 2000). The term ‘moyamoya syndrome’ refers to non-japanese patients with unilateral or bilateral terminal ICA stenosis or occlusion with basal collaterals whether idiopathic or secondary (Scott & Smith 2009). Children with neurofibromatosis, sickle cell disease, trisomy 21, or history of central nervous system radiation have an increased risk for developing moyamoya (secondary moyamoya) (Scott & Smith 2009). Symptomatic unilateral cases demonstrate the same pathology and are amenable to similar diagnostic and therapeutic approaches while the majority appear to progress to become bilateral although stabilisation is also well documented (Hirotsune et al. 1997; Seol et al. 2006b). In general the presence of moyamoya arteriopathy is associated with an increased risk of stroke recurrence a finding supported by several studies (Braun et al. 2009; Ganesan et al. 2006). Familial cases

and ethnic differences support a genetic basis for moyamoya but no single causal gene has yet been identified (Ikeda et al. 1999; Seol et al. 2006a). Histopathology studies in moyamoya show fibro-cellular intimal thickening without evidence of atherosclerosis, inflammation or emboli (Hosoda et al. 1997). Endothelial cell dysfunction and dysregulation of growth factors has been postulated as a possible mechanism of disease (Yamamoto et al. 1998; Yoshimoto et al. 1996). Given that haemodynamic failure is responsible for cerebral ischaemia in the majority of cases, a variety of surgical interventions have been used to improve perfusion in this group of children. Procedures can broadly be considered in two groups: direct extracranial-intracranial (EC-IC) bypass and indirect revascularisation procedures (Fung et al. 2005; Golby et al. 1999). The optimal surgical technique remains controversial (Fung, Thompson, & Ganesan 2005; Golby et al. 1999).

#### **1.2.2.5 Other arteriopathies**

Several other arteriopathies are encountered in childhood AIS some of which are discussed below. Fibro-muscular dysplasia (FMD) is a nonatherosclerotic, noninflammatory disease characterised by focal hyperplasia of blood vessels throughout the arterial tree, but often involving cervico-cranial vascular network, most commonly the internal carotid artery (Slovut and Olin 2004). On catheter angiography, FMD vessels may have a classic 'string of beads' appearance, or show a smooth focal stenosis or long tubular stenosis (Osborn and Anderson 1977). The prevalence of symptomatic renal FMD in the general population is estimated to about 4 / 1000 and cervico-cephalic FMD is probably half as common as renal FMD (Begelman and Olin 2000; Slovut & Olin 2004). Renal FMD can lead to hypertension

and progressive renal atrophy (Begelman & Olin 2000; Slovut & Olin 2004). Cervico-cephalic FMD can result in ischaemic or haemorrhagic stroke, cervical artery dissection and may be associated with intracerebral aneurysms, with risk of subarachnoid haemorrhage (Begelman & Olin 2000; Slovut & Olin 2004).

Neurofibromatosis type 1 (NF1) is associated with intracerebral arteriopathies (Rea et al. 2009). Rea et al. recently showed that arteriopathy was more common in patients with NF1 and optic gliomas (Rea et al. 2009). Follow-up at a mean of 7 years after diagnosis of arteriopathy showed that 35% had progressive arteriopathy requiring revascularization surgery (Rea et al. 2009).

Loeys-Dietz syndrome (LDS) is caused by mutation in the genes encoding the transforming growth factor beta receptor 1 and 2 (*TGFBRI* and *TGFRB2*) and is characterised by arterial tortuosity and aneurysms, hypertelorism, and bifid uvula or cleft palate (LeMaire et al. 2007; Pagon et al. 2008; Singh et al. 2006). The arterial disease is widespread and can involve all aortic segments and major branching arteries, necessitating cardiovascular imaging beyond the aortic root segment (LeMaire et al. 2007; Pagon et al. 2008; Singh et al. 2006). Cerebral/cervical involvement includes dissection of the carotid and vertebrobasilar arteries and intracranial aneurysms (Pagon et al. 2008).

Arterial tortuosity syndrome (ATS) is a rare autosomal recessive connective tissue disorder characterized by tortuosity and elongation of the large and medium-sized arteries, propensity to aneurysms formation, vascular dissection, and pulmonary arteries stenosis (Callewaert et



al. 2008; Coucke et al. 2006; Satish et al. 2008). Other typical manifestations are dysmorphic features, hyperextensible skin, cutis laxa, herniae, skeletal abnormalities, joints hypermobility, and congenital contractures (Callewaert et al. 2008; Coucke et al. 2006; Satish, Nampoothiri, & Kappanayil 2008). ATS is due to mutations in *SLC2A10* gene, located on chromosome 20q13.1 and encoding for the 541 amino acid facilitative glucose transporter 10 (*GLUT10*), consisting in 12 transmembrane hydrophobic segments connected by 5 intracellular and 6 extracellular loops (Callewaert et al. 2008; Coucke et al. 2006; Satish, Nampoothiri, & Kappanayil 2008).

Aicardi–Goutieres syndrome (AGS) is a genetically determined encephalopathy, which, in its classical presentation, shows phenotypic overlap with the sequelae of congenital infection. It is a genetically heterogeneous disorder caused by mutations in any of the genes encoding the 3' to 5' exonuclease *TREX1* (AGS1), the three non-allelic components of the *RNASEH2* endonuclease complex (AGS2, 3, and 4) and the *SAMHD1* protein (AGS5) (Crow et al. 2006a; Crow et al. 2006b; Rice et al. 2009). Xin et al. describe an autosomal recessive condition characterized with cerebral vasculopathy and early onset of stroke caused by mutations in *SAMHD1* (Xin et al. 2011). Cerebral vasculopathy was a major hallmark of the condition with a common theme of multifocal stenoses and aneurysms in large arteries, accompanied by chronic ischaemic changes, moyamoya morphology, and evidence of prior acute infarction and haemorrhage (Xin et al. 2011). Early signs of the disease included mild intrauterine growth restriction, infantile hypotonia, and irritability, followed by failure to thrive and short stature (Xin et al. 2011). Acrocyanosis, Raynaud's phenomenon, chilblain lesions, low-pitch hoarse voice, glaucoma, migraine headache, and arthritis were frequently

observed (Xin et al. 2011). Ramesh et al. have also reported on 5 patients with a clinical diagnosis of AGS who developed intracranial large artery disease that was primarily occlusive. All patients were shown to harbor mutations biallelic mutations in *SAMHDI* (Ramesh et al. 2010).

The vascular smooth muscle cell (SMC)-specific isoform of  $\alpha$ -actin (*ACTA2*) is a major component of the contractile apparatus in SMCs located throughout the arterial system (Milewicz et al. 2010; Munot et al. 2011a). Heterozygous *ACTA2* mutations cause familial thoracic aortic aneurysms and dissections (TAAD), but only half of mutation carriers have aortic disease (Guo et al. 2007). Linkage analysis and association studies of individuals in 20 families with *ACTA2* mutations indicate that mutation carriers can have a diversity of vascular diseases, including premature onset of coronary artery disease and premature ischaemic strokes (including Moyamoya disease) as well as previously defined TAAD (Guo et al. 2007). Sequencing of DNA from patients with nonfamilial TAAD and from premature-onset CAD patients independently identified *ACTA2* mutations in these patients and premature onset strokes in family members with *ACTA2* mutations (Guo et al. 2007).

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL) remains the most common hereditary small vessel disease caused by >190 different mutations in the *NOTCH3* gene on chromosome 19 which encodes a transmembrane receptor primarily expressed in arterial smooth muscle cells (SMCs) involved in the *NOTCH* signaling pathway (Joutel et al. 1996). Mutant *NOTCH3* instigates degeneration of vascular smooth muscle cells in small arteries and arterioles leading to

recurrent lacunar infarcts (Joutel et al. 1996). The most common clinical manifestations are migraine headaches and transient ischaemic attacks or strokes, which usually occur between 40 and 50 years of age, although MRI is able to detect signs of the disease years prior to clinical manifestation of disease (Choi 2010; Fattapposta et al. 2004; Munot, Crow, & Ganesan 2011a). Whilst the predominant cerebrovascular manifestations are in a small vessel distribution, large artery disease has also been observed (Choi et al. 2005). Genetically confirmed CASADIL has also been described in the paediatric population (Hartley et al. 2010).

The *COL4A1* gene encodes the alpha-1 chain of type IV collagen, the main constituent of basement membranes widely expressed in all tissues, including vascular beds (Munot, Crow, & Ganesan 2011a; Plaisier et al. 2007). Mutant *COL4A1* likely disrupts the extracellular matrix resulting in fragile vessel walls (Munot, Crow, & Ganesan 2011a; Plaisier et al. 2007). A wide range of phenotypes with overlapping systemic and central nervous system features have been described in association with *COL4A1* mutations, including idiopathic cerebral small-vessel disease in children (Plaisier et al. 2007; Shah et al. 2010). Occlusive and aneurysmal cerebral arteriopathies, resulting in ischaemic and haemorrhagic stroke can occur in the same family (Gould et al. 2006; Plaisier et al. 2007).

PHACE(S) is a neurocutaneous disorder comprising a cervicofacial haemangioma with one or more of a posterior fossa malformation, arterial, cardiac and eye abnormalities or a sternal pit (Heyer et al. 2008). The arteriopathy of PHACES includes dysplasia or hypoplasia as well as steno-occlusive disease (Heyer et al. 2008).

### **1.2.2.6 Imaging evaluation of cerebral arteriopathies**

The majority of children presenting with an acute AIS demonstrate non-atherosclerotic intra- or extracranial cerebral arteriopathies (Fullerton, Wu, Sidney, & Johnston 2007; GANESAN, Prengler, Wade, & Kirkham 2006; Mackay et al. 2011). Given the importance of arterial disease in the aetiology of childhood AIS accurate and timely detection are clearly key issues (Fullerton, Wu, Sidney, & Johnston 2007; Ganesan, Prengler, Wade, & Kirkham 2006; Mackay et al. 2011). Currently, there are several imaging modalities that can be employed for the evaluation of the cerebral circulation, namely ultrasound, magnetic resonance angiography (MRA) with or without contrast, computed tomography angiography (CTA) and catheter cerebral angiography (CA)(Jones, Ganesan, Saunders, & Chong 2010; Roach et al. 2008). Arterial disease is most commonly intracranial but may be cervical and thus an adequate examination should include the arterial circulation from the aortic arch to the circle of Willis (Fullerton, Wu, Sidney, & Johnston 2007; Ganesan, Prengler, Wade, & Kirkham 2006; Mackay et al. 2011). Ultrasound has relatively limited applications in paediatric AIS, other than in screening and follow-up of children with sickle cell disease (Adams et al. 1992; Kenton et al. 1997). Of note, early or small parenchymal lesions and lesions located in the posterior fossa can be missed with CT scans (Hinshaw Jr et al. 1980; Jones, Ganesan, Saunders, & Chong 2010). 3D time-of-flight (TOF) MRA is commonly used for detecting intracranial stenotic or occlusive disease (Husson et al. 2002). MRA can be combined with cross sectional imaging of the brain with MRI – the latter is usually required in order to differentiate between AIS and stroke mimics (Hunter 2002; Husson et al. 2002). However TOF is insensitive to slow flow or in-plane flow which leads to overestimation of the severity and length of a stenosis especially when there is dephasing

secondary to turbulence (Aviv et al. 2006; Eleftheriou et al. 2010; Rollins et al. 2000). Of note, the spatial resolution of MRA also limits its ability to visualise medium and small sized arteries (Aviv et al. 2006; Eleftheriou et al. 2010; Rollins, Dowling, Booth, & Purdy 2000). Contrast enhanced MRA may help overcome these difficulties (Ishimaru et al. 2007; Kuker et al. 2008). Ishimaru et al. evaluated the use of 3D TOF MRA with and without contrast in comparison to CA, in a small series of adult patients with acute ischaemic stroke and found that post contrast 3D TOF MRA more accurately delineated the extent of stenotic or occlusive arterial disease (Ishimaru et al. 2007). Cases of AIS with normal MRA are well described in the literature (Jones, Ganesan, Saunders, & Chong 2010; Munot et al. 2011b). Munot et al. recently reported on a series of forty children with AIS and normal MRA (Munot et al. 2011b). Although similar in terms of age and gender to those with abnormal MRA, children with normal MRA were significantly more likely to have at least 1 other risk factor ( $P=0.012$ ) while abnormal MRA was significantly associated with clinical recurrence ( $P=0.001$ ) (Munot et al. 2011b). Notably however CA imaging findings are not discussed in this study.

CTA is increasingly used in adults with ischaemic stroke and appears to be more accurate than MRA for delineating intracranial stenosis and occlusion, especially in the posterior circulation (Knauth et al. 1997; Roach et al. 2008; Shrier et al. 1997). The disadvantages of CTA in children include exposure to radiation and the need for intravenous contrast (Jones, Ganesan, Saunders, & Chong 2010; Roach et al. 2008). CTA has an evolving role in the evaluation of children with intracranial haemorrhage but has not been evaluated in comparison with either MRA or CA in childhood AIS (Demchuk et al. 2012; Roach et

al.2008). In general most children with AIS can be investigated with MRA combined with MRI. CTA may have a role in providing further endoluminal visualisation of a specific area of concern but is unlikely to supersede CA in the near future (Borisch et al. 2007; Chamoun et al. 2008; Knauth, von Kummer, Jansen, & Sartor 1997; Roach et al. 2008; Shrier et al. 1997).

CA remains the gold standard for cerebrovascular imaging, especially for the evaluation of the posterior circulation and the distal intracranial cerebral arterial circulation (Aviv et al. 2006; Eleftheriou et al. 2010; Rollins, Dowling, Booth, & Purdy 2000). CA also provides dynamic information about the cerebral circulation (Aviv et al. 2006; Eleftheriou et al. 2010; Rollins, Dowling, Booth, & Purdy 2000). Rollins et al. compared MRA and CA findings in 18 children with idiopathic AIS and reported a positive predictive value of MRA for arteriopathy of 100%, with a negative predictive value of 88%. MRA was equivalent to CA in the detection and depiction of proximal MCA disease (Rollins, Dowling, Booth, & Purdy 2000); however, delineation of disease in the ICA and detection of peripheral embolic disease were better with CA than MRA (Rollins, Dowling, Booth, & Purdy 2000). Aviv et al. reported only a fair correlation between CA and MRA lesion identification in 25 children with cPACNS (Aviv et al. 2006). Most of the lesions identified on CA and not MRA within the distal, posterior circulation. Similarly we have previously reported on moderate agreement between the two modalities ( $\kappa=0.51$  95%CI 0.37-0.66) in a case series of 14 children with suspected CNS vasculitis (Eleftheriou et al. 2010). The risk of complications of CA appears to be very low in expert hands (Aviv et al. 2006; Eleftheriou et al. 2010). In general, despite the non-invasive modalities described above, CA has a continuing role in the

investigation of childhood AIS, in particular where infarction affects more than one arterial territory or is confined to small vessel distribution (to look for evidence of vasculitis), where infarction is in the posterior circulation territory (to look for vertebral dissection), in moyamoya when revascularisation is being considered and in other arteriopathies which might be amenable to endovascular interventions (e.g. angioplasty or stenting) (Aviv et al. 2006; Eleftheriou et al. 2010; Rollins, Dowling, Booth, & Purdy 2000). In addition, some conditions, including extracranial arterial dissections, particularly involving the posterior circulation, and small-vessel vasculitis, are difficult to exclude on MRA (Jones, Ganesan, Saunders, & Chong 2010; Roach et al. 2008).

### ***1.2.3 Cardiac abnormalities***

Cardiac procedures such as heart surgery, catheterization, and extracorporeal membrane oxygenation (ECMO) are all well documented risk factors for childhood AIS, likely mediated through a mechanism that involves cardioembolism (Fallon et al. 1995; Jonas 1998; Segal et al. 2001). In addition, congenital heart disease including valvular heart disease, cardiac arrhythmias, and cardiomyopathy are also associated with childhood AIS regardless of invasive cardiac procedures (Kumar 2000; Phornphutkul et al. 1973). Aberrant blood flow and thrombosis that can embolize to the cerebral vessels are possibly implicated in AIS in the presence of these risk factors. Lo et al. actually reported that congenital heart disease was the most frequent comorbidity in children with AIS in their study (Lo et al. 2009). Of note, nearly 25% of children diagnosed with AIS in the Canadian Registry had cardiac disease at presentation (Fullerton, Wu, Sidney, & Johnston 2007). Similarly,

Ganesan et al. reported that 28% of children in their UK based childhood AIS cohort study had cardiac abnormalities (Ganesan et al. 2003).

Moreover, it remains uncertain if a patent foramen ovale (PFO) is a significant risk factor for childhood AIS. PFO is the most commonly persistent abnormality of fetal origin, with prevalence on echocardiography of 15–25% in the general population (Di Tullio et al. 2007). Although the presence of PFO has been associated with an increased risk of AIS in studies of young adults a causative relationship has not been established (Mas et al. 2001). It is not known whether children with cryptogenic AIS have a similarly increased incidence of PFO relative to healthy children (Benedik et al. 2011; Kirkham et al. 2011). However, PFO is more common in children than in adults and thrombosis within the venous system (the putative source of embolism) much less so (Benedik, Zaletel, Megli-ì, & Podnar 2011; Kirkham, Salmon, & Khambadkone 2011). At present the evidence base to recommend PFO closure in children with AIS and a right-to-left shunt intracardiac shunt is lacking (Kirkham, Salmon, & Khambadkone 2011). However, extrapolation from adult data suggests there may be a place for PFO closure in selected children with cryptogenic AIS and a significant right to left shunt, with no other risk factors but the decision should be carefully considered on a case by case basis (Kirkham, Salmon, & Khambadkone 2011).



#### **1.2.4 Sickle cell disease**

Sickle cell disease (SCD) is the most common cause of childhood stroke in certain parts of the world (Kirkham 2007). SCD is a disorder caused by elevations of intraerythrocyte and total blood viscosity (Verduzco and Nathan 2009). Hypoxia induces gelation of haemoglobin S (HbS) that deforms the erythrocyte and its membrane and causes cation loss and increased erythrocyte surface expression of adhesion molecule receptors (Verduzco & Nathan 2009). This results in obstructive adhesion of sickle cells to each other and to vascular endothelium (Verduzco & Nathan 2009). The obstruction and inflammation in turn cause further hypoxia and acidosis and, consequently, further sickling (Verduzco & Nathan 2009). Approximately 11% of SCD patients have clinically apparent strokes before the age of 20 (Ohene-Frempong et al. 1998). That risk increases to 24% by the age of 45 (Ohene-Frempong et al. 2009). The risk of stroke is highest during the first decade, and it is most significant between ages 2 and 5, when it reaches 1.02% per year (Ohene-Frempong et al. 1998). The risk is lowest before the age of 2, probably because of the protective influence of fetal haemoglobin on sickling (Ohene-Frempong et al. 2009). A child with SCD has a stroke risk that is 333 times greater than that of a healthy child without SCD or heart disease (Broderick et al. 1993; Ohene-Frempong et al. 1998; Verduzco & Nathan 2009). In children with “abnormal” transcranial doppler (noninvasive ultrasound that allows real-time evaluation of the intracranial cerebral circulation) studies, the risk is more than 3000 times greater (Adams et al. 1997; Verduzco & Nathan 2009). Silent infarcts defined as increased signal intensity on T2-weighted MR images and no history of physical findings of a focal neurologic deficit lasting more than 24 hours, are the most frequent type of neuroischaemic damage in SCD, occurring in

approximately 20% of the patients without overt stroke by 14 years of age and are associated with significant cognitive deficits (Moser et al. 1996; Pavlakis et al. 1988).

SCD is commonly associated with a progressive occlusive arteriopathy (a secondary form of moyamoya syndrome) that involves the internal carotid arteries and proximal middle cerebral arteries, with relative sparing of the posterior circulation (Stockman et al. 1972; Verduzco & Nathan 2009). Moyamoya morphology is a risk factor for subsequent strokes (Dobson et al. 2002). Other risk factors for stroke in SCD include hypertension, raised peripheral blood white cell count, previous transient ischaemic attacks, priapism, acute anaemia, recent acute chest syndrome, or history of transfusion within the past 2 weeks (Ohene-Frempong et al. 1998). In addition a recent case-control study reported an increased incidence of PFO in these patients (Dowling et al. 2010).

Of note although stroke risk in children with SCD is high, this is the only case where there is a proven method for primary stroke prevention (Adams et al. 1998 ;Adams and Brambilla 2005; Roach et al. 2008). In the Stroke Prevention Trial in Sickle Cell Anaemia children 2 to 16 years of age with HbSS who had no prior stroke history but an “abnormal” transcranial Doppler value were randomly assigned to either chronic blood transfusions every 3 to 4 weeks (goal HbS <30%) or standard care (Adams et al. 1998). The trial was terminated early when 12 strokes occurred, 11 of which were in the standard care group (Adams et al. 1998). The introduction of chronic transfusions for primary stroke prevention ever since had an immediate impact on stroke rates that dropped by a factor of 5 within 2 years (Fullerton et al. 2004). In addition, the Primary Stroke Prevention in Sickle Cell Anemia (STOP II) trial data

suggest that lifelong blood transfusions may be necessary, as there is a high risk of recurrence relatively soon after transfusion cessation (Adams & Brambilla 2005).

### ***1.2.5 Genetic and acquired thrombophilias as risk factors for AIS***

As thrombo-embolic phenomena prevail in the pathophysiology of childhood AIS, investigation of the contribution of thrombophilias to risk of incident and recurrent AIS in childhood is crucial. Thrombophilia refers to a systemic predisposition to thrombosis and may be genetic or acquired (Kenet et al. 2010; Trenor III and Michelson 2010; Walker et al. 2001).

#### **1.2.5.1 Genetic thrombophilias**

An increased frequency of heterozygous factor V Leiden (FVL) polymorphism in childhood-onset AIS cases as compared to healthy controls has been demonstrated by several studies (Duran et al. 2005; Kenet et al. 2000; Nowak-Gottl et al. 1999). In the study by Nowak et al. approximately 20% of AIS cases were heterozygous for FVL, as compared to 4% of controls (OR 6; 95% CI 2.97-12.1) (Nowak-Gottl et al. 1999). In addition the prothrombin G20210A polymorphism was found in 6% of patients as compared to 1 % of controls (OR 4.7; 95% CI 1.4 to 15.6) (Nowak-Gottl et al. 1999). Furthermore, protein S and protein C deficiency have also been reported in association with AIS in several case reports and case control studies (Brown et al. 1993; Haywood et al. 2005; Nowak-Gottl et al. 1999). In a cohort series of 310 German children protein C deficiency was confirmed as a relative risk of recurrent childhood-onset AIS of 3.5 (95% CI 1.1-10.9) (Sträter et al. 2002a). The same

series reported on a high recurrence risk for AIS for children with elevated lipoprotein (a) (RR=4.4, 95% CI=1.9-10.5) (Sträter et al. 2002a). Furthermore, the recent meta-analysis of Kenet et al. demonstrated that the MTHFR C677T mutation was found more frequently in childhood AIS than in healthy controls; however, it is uncertain whether this serves as an independent risk factor or in relation to plasma homocysteine levels (Kenet et al. 2010). Of note, Alsayouf et al. also analyzed the potential link between MTHFR 677C>T homozygosity and childhood stroke demonstrating that there was no difference in the prevalence of the MTHFR 677C>T mutation in the studied cohort compared with the general population (Alsayouf et al. 2011). In addition, the Kenet et al. meta-analysis confirmed many of the associations discussed above (Kenet et al. 2010). Specifically a significant association with AIS for FVL (OR 3.70; CI 2.82–4.85), factor II G20210A (OR 2.60; 95% CI 1.66–4.08), protein C deficiency (OR 11.0; 95% CI 5.13-23.59), lipoprotein (a) (OR 6.53; 95% CI 4.46–9.55) and MTHFR C677T mutation (OR 1.58; 95% CI 1.20–2.08) was demonstrated (Kenet et al. 2010). Of note however this meta-analysis summarised data on both studies of childhood-onset AIS and neonatal AIS. Furthermore, the relationship between the timing of the cerebrovascular event and the timing of the blood sample for thrombophilia testing is not specified in most studies discussed. This is an important confounding factor because thrombosis can, for instance, result in transient, acquired low levels of antithrombin, protein C, and/or protein S (Trenor III & Michelson 2010). Lastly widely varying results between the above discussed studies are likely to reflect ethnic differences between studied cohorts.

### **1.2.5.2 Acquired thrombophilias**

One retrospective case-control study demonstrated elevated levels of factor VIII activity in 65% of childhood AIS patients tested four months after incident stroke as compared to 12.5% of controls (Cangöz et al. 2004). Sträter et al. demonstrated that the prevalence of anticardiolipin antibodies (IgG or IgM) was 10% in a group of children with cardioembolic AIS (Sträter et al. 1999). Another case-control study reported a greater than 6-fold increase in the odds of stroke in patients with antiphospholipid antibodies (aPL; lupus anticoagulant or anticardiolipin antibodies) in childhood AIS cases as compared to healthy controls (Kenet et al. 2000). In the Kenet et al. meta-analysis, a pooled examination of aPL antibodies of multiple types demonstrated a significant association between aPL antibodies and childhood AIS (neonates included-OR 6.95; CI 3.67-13.14) (Kenet et al. 2010). Of note however, transient aPL are commonly found after even mild viral infections and it is unknown whether these transient antibodies confer an increased thrombotic risk to these children (Bernard et al. 2011). More recently other coagulation cascade activation markers have been proposed as prognostic factors in childhood AIS (Bernard et al. 2010; Bernard, Manco-Johnson, & Goldenberg 2011). A recent cohort study has demonstrated that D-dimer (a marker of ongoing coagulation activation) is acutely elevated in patients with childhood-onset AIS, particularly patients with cardioembolic stroke (Bernard et al. 2010). Perhaps in many cases, transient acquired risk factors particularly in the setting of systemic infection may be more potent than modest genetic thrombophilias.

### **1.3 Infection and inflammation in the pathogenesis of cerebral arteriopathies and childhood arterial ischaemic stroke**

A wide range of mechanisms have been suggested to link inflammation and infection to the pathophysiology of ischaemic stroke (Emsley and Tyrrell 2002; Macko et al. 1996a; McColl et al. 2009). As a general response to tissue injury, inflammation is ubiquitous (Emsley & Tyrrell 2002; Frijns and Kappelle 2002; Sullivan et al. 2000; Zoppo et al. 2000). It is now well established that one of the fundamental features of the immune response is an inflammatory cascade that can also cause extensive damage to host tissues (Emsley & Tyrrell 2002; Frijns & Kappelle 2002; Sullivan, Sarembock, & Linden 2000; Zoppo et al. 2000). In that context inflammation is a major contributing factor to many vascular events, including atherosclerotic plaque development and rupture, aortic aneurysm formation, angiogenesis, and ischaemia/reperfusion injury (Emsley & Tyrrell 2002; Libby et al. 2002; Romagnani et al. 2004; Sullivan, Sarembock, & Linden 2000). This immune response is mediated by both circulating and resident leukocytes and a number of cells that the leukocytes interact with such as vascular endothelium and smooth muscle cells (Frijns & Kappelle 2002; Sullivan, Sarembock, & Linden 2000). In addition, the process involves up regulation of a series of adhesion molecules on leukocytes and underlying vascular endothelium and the release of various pro-inflammatory cytokines, and chemokines (Frijns & Kappelle 2002; Sullivan, Sarembock, & Linden 2000). In acute stroke there are several areas in which inflammation may be important: in association with underlying vascular disease predisposing to stroke; as a precipitating factor triggering an acute event; once a stroke has occurred, as an acute response to tissue injury, perhaps exacerbating the injury or

its consequences; or as a prognostic marker for recurrent cardiovascular events (Emsley & Tyrrell 2002; McColl, Allan, & Rothwell 2009). Notably atherosclerosis is now widely considered an inflammatory disease while multiple mechanisms have been proposed to link inflammatory stimuli to the development and progression of atherosclerotic plaque (Emsley & Tyrrell 2002; Hegele 1996; Libby, Ridker, & Maseri 2002). These involve a direct effect of C-reactive protein to human arterial endothelial cells with a subsequent recruitment of inflammatory cells, release of cytokines such as TNF- $\alpha$  and IL-1 and smooth muscle cell proliferation fibrous plaque formation (Emsley & Tyrrell 2002; Hegele 1996; Libby, Ridker, & Maseri 2002).

Inflammation is also a main feature of the host defence response to infection and is necessary for the containment and removal of any pathogen as well as for promoting tissue repair (Croizat et al. 2009; McColl, Allan, & Rothwell 2009). However, in some cases, there appears to be a disruption of the equilibrium between the beneficial effects of infection induced inflammation and the potential aggravation of inflammatory pathways that predispose to and/or exacerbate acute ischaemic brain injury and accelerate the progression of arterial disease (Croizat, Vivier, & Dalod 2009; McColl, Allan, & Rothwell 2009). In adult stroke, numerous clinical studies have suggested a link between systemic infection and increased stroke susceptibility (Cook et al. 1998; Heuschmann et al. 2001; McColl, Allan, & Rothwell 2009; Ridker et al. 1998). In patients with bacteraemia, thromboembolic complications are relatively common, occurring for instance in approximately 20% of patients with infective endocarditis (Valtonen et al. 1993). Tuberculosis (TB) has also been associated with cerebral infarction mainly in the anterior circulation although a few reported

cases involved the posterior territories (Leiguarda et al. 1988; Sheu et al. 2010). A progressive vasculopathy has been described in TB meningitis with evidence of stenosis or spasm initially followed by progressive occlusion that may result in re-infarction (Leiguarda et al. 1988; Sheu et al. 2010). In addition, *Borellia burgdorferi* can penetrate the blood-brain barrier and cause neuroborreliosis or trigger vasculitis (Logigian et al. 1990). In malaria cortical haemorrhagic infarcts relating to generalized cerebral oedema and reduced cerebral perfusion are well described (Brewster et al. 1990). Several chronic infectious pathogens such as *Chlamydia pneumonia* and *Helicobacter Pylori*, acute bacterial and viral infections have been associated with an increased risk of cerebrovascular disease in adults (Cook et al. 1998; Heuschmann et al. 2001; McColl, Allan, & Rothwell 2009; Ridker, Hennekens, Stampfer, & Wang 1998). In general these infections appear to induce a transient state of increased stroke susceptibility, particularly in the weeks following infection (Emsley & Tyrrell 2002; McColl, Allan, & Rothwell 2009). The greatest risk period after infection coincides with the high-level pro-inflammatory phase of the immune response suggesting that acutely elevated systemic inflammation maybe a stroke trigger (Emsley & Tyrrell 2002; McColl, Allan, & Rothwell 2009).

Infection may also promote a shift toward a pro-thrombotic tendency by creating an imbalance in coagulation and fibrinolysis (Ameriso et al. 1991; Emsley & Tyrrell 2002; Macko et al. 1996b). A prothrombotic state has been described in inflammation and infection-associated stroke (Ameriso, Wong, Quismorio, & Fisher 1991; Emsley & Tyrrell 2002; Macko et al. 1996b). Examples of components of the coagulation system that are modified in inflammation or infection-associated stroke include elevated C4b-binding



protein, reduced circulating antithrombotic activated protein C, and a low ratio of tissue plasminogen activator to plasminogen activator inhibitor (Emsley & Tyrrell 2002; Macko et al. 1996b; McColl, Allan, & Rothwell 2009). Increased fibrin D-dimer levels and fibrinogen levels also occur in patients with acute ischaemic stroke in the context of systemic infection (Ameriso, Wong, Quismorio, & Fisher 1991; Emsley & Tyrrell 2002; McColl, Allan, & Rothwell 2009). Furthermore microvascular obstruction may also contribute to a reduction in tissue perfusion and worsen cerebral ischaemia (Emsley & Tyrrell 2002). Increased levels of CRP that promotes localized coagulation, and therefore thrombosis, by stimulating monocytes to produce tissue factor, additionally contribute to a prothrombotic state (Cermak et al. 1993a; Emsley & Tyrrell 2002). Of note elevated circulating IL-6 levels in patients with acute ischaemic stroke have been associated with decreased levels of free protein S (Emsley & Tyrrell 2002; Vila et al. 2000). In addition, endotoxins, other bacterial toxins, and proinflammatory cytokines such as IL-1 and TNF- $\alpha$  may well contribute to thrombosis via effects on endothelial function (Emsley & Tyrrell 2002; Yudkin et al. 1999).

Augmentation of endothelial dysfunction by infections could further increase susceptibility to stroke (Emsley & Tyrrell 2002; Oshima et al. 2005; Prasad et al. 2002). A link between chronic infections, an inflammatory state, and endothelial dysfunction has been reported by several studies in adults (Emsley & Tyrrell 2002; Oshima et al. 2005; Prasad et al. 2002; Vallance et al. 1997). In children Charakida et al. have shown that acute infection in childhood is associated with impaired endothelium-dependent vasodilation suggesting that even a minor infectious stimulus in childhood relevant to normal daily life is associated with vascular dysfunction (Charakida et al. 2005).

Infection and inflammation have also been implicated in the pathophysiology of childhood AIS (Amlie-Lefond et al. 2009; Amlie-Lefond and Fullerton 2010; Askalan et al. 2001; Fullerton et al. 2011; Sebire et al. 1999). In particular, the link between childhood AIS and infection was first proposed because of the temporal association with varicella zoster infection (Askalan et al. 2001; Sebire, Meyer, & Chabrier 1999). Sebire et al. reported on a case control study of children with AIS, showing that 64% of children who had strokes had varicella within the 9 month period prior to stroke versus 9% in the control group (Sebire, Meyer, & Chabrier 1999). Furthermore, Askalan et al. in 2001 reported on a prospective cohort study conducted in young children (aged 6 months to 10 years) with AIS at 2 Canadian institutions, showing that 22 (31%) of 70 consecutive children with AIS had a varicella infection in the preceding year compared with 9% in the healthy population (Askalan et al. 2001). Children in the varicella cohort were more likely to have basal ganglia infarcts ( $P<0.001$ ), abnormal cerebral vascular imaging ( $P<0.05$ ), and recurrent AIS or TIAs ( $P<0.05$ ) than those in the non-varicella cohort (Askalan et al. 2001). The authors concluded that the 31% incidence of varicella within the preceding year in their paediatric AIS cohort represents a 3-fold increase over the 9% annual incidence of varicella in Canadian children (Askalan et al. 2001). Of note, however, no varicella studies (anti VZV IgG or VZV DNA PCR in either peripheral blood or cerebrospinal fluid testing) were systematically reviewed in any of these cohorts. Furthermore, Braun et al. demonstrated recently that stroke was preceded by chickenpox in 44% of children with unilateral cerebral arteriopathies within one year period prior to stroke (Braun et al. 2009).

The underlying mechanism for VZV vasculopathy causing AIS remains unknown. Multiple mechanisms have been proposed including a reactivation of latent infection housed in the cranial nerve and dorsal root ganglia, with migration of VZV towards the anterior cerebral circulation causing direct infection of cerebral arteries, and possibly leading to thrombosis, necrosis, dissection and aneurysm (Gilden et al. 2009; Gilden et al. 2000; Mueller et al. 2008; Nagel et al. 2010). Animal studies have identified afferent fibers from trigeminal and dorsal root ganglia to both intracranial and extracranial blood vessels, providing an anatomic pathway for possible trans-axonal spread of virus (Mayberg et al. 1981). In addition pathological and virological analyses of cerebral arteries from adults patients who died from VZV vasculopathy have revealed herpes virions, VZV antigen and VZV DNA in the walls of cerebral arteries (Gilden, Cohrs, Mahalingam, & Nagel 2009; Nagel et al. 2008; Nagel, Mahalingam, Cohrs, & Gilden 2010; Nagel et al. 2011). Nagel et al. recently reported on a series of 30 patients with VZV vasculopathy and demonstrated cerebro-spinal fluid (CSF) pleocytosis in 20 (67%), and imaging abnormalities in 29 (97%) (Nagel et al. 2008). Angiography revealed abnormalities in 70% of the patients who had vascular imaging (Nagel et al. 2008). Large and small arteries were involved in 50% of studied cases. CSF of 9 (30%) patients contained VZV DNA while 28 (93%) had anti-VZV IgG antibody in CSF; in each of these patients, reduced serum/CSF ratio of VZV IgG confirmed intrathecal synthesis (Nagel et al. 2008). In addition the same group reported on their findings on histological and by immunohistochemical examination of normal and VZV-infected cerebral and temporal arteries in 3 patients with VZV vasculopathy (Nagel et al. 2011). They showed that all VZV-infected arteries contained a disrupted internal elastic lamina; a hyperplastic intima composed of cells expressing  $\alpha$ -smooth muscle actin and smooth muscle myosin

heavy chain but not endothelial cells expressing CD31; and decreased medial smooth muscle cells (Nagel et al. 2011). The finding of VZV presence primarily in the adventitia early in infection and in the media and intima later supports the theory that after reactivation from ganglia, VZV spreads transaxonally to the arterial adventitia followed by transmural spread of virus (Nagel et al. 2011). The authors suggested that stroke in VZV vasculopathy may result from changes in arterial caliber and contractility produced in part by abnormal accumulation of smooth muscle cells and myofibroblasts in thickened neointima and disruption of the media (Nagel et al. 2011). Another potential cofactor that has emerged in VZV-associated AIS is the role of transient auto-antibodies to phospholipids and coagulation cascade activation during or after varicella infection (Josephson et al. 2001; Manco-Johnson et al. 1996; Regnault et al. 2005). Both the presence of lupus anticoagulant and a transient protein S deficiency were found in a child with disseminated intravascular coagulation during varicella (Josephson et al. 2001; Manco-Johnson et al. 1996; Regnault et al. 2005).

Furthermore, multiple other infectious agents have also been linked to case reports of arteriopathies and stroke in children such as enterovirus, *Mycoplasma pneumoniae* and *Parvovirus B19* (Fu et al. 1998; Guidi et al. 2003; RibaI et al. 2003). *Borrelia* infection has also been reported in association with childhood AIS (Cox et al. 2005). Cerebral infarction is not uncommon in meningitis particularly in children under the age of 1 year old with streptococcus pneumonia and salmonella (Chang et al. 2003). *Haemophilus influenza* was also described as a cause of acute stroke in 5% of patients in the pre-vaccination era (Kerr and Filloux 1992; Taft et al. 1986). Various types of cerebrovascular disease including aneurysms and occlusive disease have been reported in association with HIV1 infection

either at presentation or during the course of the disease (Dubrovsky et al. 1998; Leeuwis et al. 2007). In addition Bandaru et al. have recently reported on a cohort of 42 children with AIS at a single centre examining the role of *Chlamydia Pneumoniae* infection as a risk factor for AIS and showed *C. pneumoniae* seropositivity (IgG) in 14 of the 42 (33.3%) stroke patients (Bandaru et al. 2010).

Strengthening this hypothesis, the IPSS an international registry of childhood AIS cases enrolled at 30 centres, reported recently on epidemiological data also suggesting an association between childhood cerebral arteriopathies and recent minor infections (Amlie-Lefond et al. 2009). Among 508 children enrolled with AIS a recent upper respiratory infection was noted in 9.1% of those with a cerebral arteriopathy, compared with 4.5% of those without an arteriopathy (OR, 2.1; P=0.048) (Amlie-Lefond et al. 2009). In this study, 25% of the children were classified as having FCA, higher in frequency than either moyamoya (22%) or dissection (20%) (Amlie-Lefond et al. 2009). This association with upper respiratory infection was stronger for children with idiopathic arterial stenosis versus those with other arteriopathy (eg, arterial dissection, moyamoya) or no arteriopathy (OR, 2.8; P=0.003; 95% CI, 1.3-6.1) (Amlie-Lefond et al. 2009). The only predictor of FCA was recent upper respiratory infection (OR, 2.81; 95% CI, 1.28 to 6.12; P=0.003), thus suggesting a possible role for burden of infection in the pathogenesis of cerebral arteriopathy in children (Amlie-Lefond et al. 2009). The Vascular Effects of Infection in Paediatric Stroke (VIPS) study is an ongoing international study conducted under the auspices of IPSS

that aims to further improve our understanding of the role of infection in the development of childhood cerebral arteriopathies and AIS (Fullerton et al. 2011).

So in summary, several reports suggest that inflammatory events outside the brain such as infection are associated with an elevated systemic inflammatory profile and may thus have an important impact on stroke susceptibility.

## **1.4 Genetics**

Current theories with regards to the aetiopathogenesis of childhood AIS suggest that perhaps one or more widely distributed infectious agents evoke an abnormal immunological response in genetically susceptible individuals, leading to the characteristic clinical presentation of the disease (Eleftheriou and Ganesan 2009; Fullerton et al. 2011; Munot, Crow, & Ganesan 2011a). In that context a number of candidate gene association studies have evaluated the significance of genetic polymorphisms in prothrombotic, inflammatory, immune mediated or metabolic pathways contributing to paediatric AIS. Voetsch et al. studied the plasma glutathione peroxidase (GPx-3)-GPx-3 gene promoter in a population of childhood stroke compared to controls (Voetsch et al. 2007). Eight novel strongly linked polymorphisms in the GPx-3 gene promoter that formed 2 main haplotypes (H1 and H2) were identified (Voetsch et al. 2007). The H2 haplotype that reduces the gene's transcriptional activity, thereby compromising gene expression and plasma antioxidant and antithrombotic activities was associated with an independent increase of AIS in children (odds ratio=2.13, 95% CI=1.23 to 4.90; P=0.027) (Voetsch et al. 2007). In addition, Wang et al investigated the

association of the genetic variants in the CTLA-4 and CD28 genes of children who suffered idiopathic AIS using a case-control design (Wang et al. 2009). A single nucleotide polymorphism (SNP), CTLA-4+49A/G located in exon 1 of the CTLA-4 gene, showed nominal association with the disease (OR 2.09, 95% CI 1.17-3.73,  $p = 0.012$ ) using allele-based analysis (Wang et al. 2009). The CD28IVS3 +17TT genotype was found to be more common in the patients than in the controls ( $P = 0.039$ , OR = 2.96, 95% CI = 1.02-8.58) (Wang et al. 2009). Furthermore Shi et al. genotyped polymorphisms in the TNF- $\alpha$  promoter region in Chinese children with idiopathic AIS and controls (Shi et al. 2009). Among totally 7 single nucleotide polymorphisms identified in the TNF- $\alpha$  promoter region, the variant of the -863C/A was associated with increased risk of AIS in that study (Shi et al. 2009). The TNF- $\alpha$  (-308) A allele has also been linked to stroke in sickle cell disease (Shi et al. 2009). Additionally, there is considerable evidence that primary moyamoya disease has a genetic basis, albeit probably polygenic (Hashikata et al. 2008; Roder et al. 2010). Approximately 10% of primary cases are familial and 80% of monozygotic twins are concordant (Hashikata et al. 2008; Roder et al. 2010). Although familial occurrence accounts for approximately 9% of moyamoya syndrome, the majority of cases are sporadic (Hashikata et al. 2010). Linkage analyses have shown associations with loci 3p24.2– p26, 6q25, 8q23, 12p12, and 17q2524 (Roder et al. 2010). Inoue et al. performed a linkage study to confirm their hypothesis that familial moyamoya disease is associated with human leukocyte antigens (HLAs) (Inoue et al. 1997). The *HLA* gene is located on chromosome 6, and a marker in chromosome 6q25 was shared by 16 of 19 Japanese families with moyamoya disease that were studied (Inoue et al. 1997). Overall however these candidate gene association studies have generally been hampered by relatively small numbers of patients studied, are relevant to certain ethnic

populations and have been seldom replicated by other groups, thus limiting the conclusions that can be drawn.

Advances in molecular genetics have enabled identification of several monogenic conditions involving the cerebral vasculature and predisposing to ischaemic and haemorrhagic strokes (Munot, Crow, & Ganesan 2011a). Even though relatively uncommon, association of childhood AIS with Mendelian genetic disorders has been recently established and may provide immediate insights into disease pathogenesis (Munot, Crow, & Ganesan 2011a). The presenting features and key neuroradiological findings in some monogenic disorders complicated by childhood AIS are summarised in table 1-3 (Munot, Crow, & Ganesan 2011a).

<b>Condition/ inheritance</b>	<b>Gene/disease mechanism of pathway implicated</b>	<b>Other features additional to stroke</b>	<b>Key neuroradiological features</b>
COL4A1 (AD)	COL4A1 (abnormal vessel-wall integrity)	Migraine, renal cysts, nephropathy, cerebral palsy, muscle cramps, cataracts, retinal tortuosity	Occlusive or aneurysmal cerebral arteriopathy; intracranial haemorrhage, including microbleeds; cerebral infarction; porencephaly



Pseudoxanthoma elasticum (AR)	ABCC6 (abnormal vascular homoeostasis)	Increased skin laxity, redundant skin folds, peau d'orange, angioid streaks, neovascularisation, haemorrhage, papular skin lesions, peripheral artery disease, visceral calcification	Lacunar infarcts, small-vessel disease, haemorrhage, white-matter signal abnormalities
ACTA2-associated disorders (AD)	ACTA-2 (smooth muscle proliferation)	Hypotonic bladder, congenital mydriasis, livedo reticularis PDA, thoracic aortic aneurysms and dissection, premature coronary artery	Occlusive disease including Moyamoya; aneurysms in other arterial beds
Neurofibromatosis type 1 (AD)	NF1 (smooth muscle proliferation)	Seizures, optic glioma, glioma, sphenoid dysplasia, scoliosis, Lisch nodules, optic gliomas, café-au-lait patches, axillary freckling, neurofibromas	White matter lesions, hamartomas
Williams syndrome (sporadic or AD)	ELN gene (encodes elastin)	Developmental delay, dysmorphism; supra-aortic stenosis in pulmonary, renal, coronary, and mesenteric vessels	Occlusive cerebral arteriopathy (including moyamoya)
CADASIL (AD)	NOTCH 3 (Notch signalling)	Migraine with aura, cognitive impairment, psychiatric features	Usually subcortical white-matter infarction though large-artery infarcts described

Alagille syndrome (AD)	JAG1(Notch signalling)	Developmental delay, butterfly vertebrae, posterior embryotoxon, dysmorphism, PDA, intra-hepatic bile-duct paucity, peripheral pulmonary stenosis	Occlusive or aneurysmal cerebral arteriopathy (including moyamoya)
CARASIL (AR)	HTRA1 (TGF $\beta$ pathway)	Dementia, psychiatric features spondylosis deformans, alopecia	White-matter signal abnormalities and lacunar lesions
Aicardi-Goutières syndrome (AR)	SAMHD1 (possibly immune response and vascular homeostasis)	Severe encephalopathy, regression, developmental delay, contracture, arthropathy, chilblains or Raynaud's phenomenon CSF lymphocytosis or raised CSF IFN- $\alpha$ (or both)	Occlusive or aneurysmal cerebral arteriopathy (including moyamoya); basal ganglia calcification, leukoencephalopathy
MOPD II (AR)	PCNT (unknown)	Motor delay, skeletal dysplasia, microcephaly, short stature, abnormal teeth, café-au-lait patches, dysmorphism, type 2 diabetes, precocious puberty	Occlusive or aneurysmal cerebral arteriopathy (including moyamoya)
Menkes disease (XLR)	ATP7A gene (abnormal response to vascular or endothelial injury due to infection, trauma, oxidative stress)	Hypotonia, developmental delay, seizures, regression, joint laxity, osteoporosis, kinky hair	Ischaemic and haemorrhagic cerebrovascular disease
Arterial tortuosity syndrome (AD)	SLC2A10/GLUT10 (Abnormal response to vascular or	Arachnodactyly, joint and skin laxity	Intracranial and extracranial dissections and

	endothelial injury due to infection, trauma, oxidative stress		aneurysms, craniosynostosis, Chiari malformation
Homocysteinuria (AR)	CBS/MHTFR genes(Vascular injury due to accumulation of abnormal metabolites)	Tall stature, dislocated lens, aortic dissection	Large vessel or lacunar strokes
Fabry's disease (XLR)	GLA (Vascular injury due to accumulation of abnormal metabolites)	Acroparaesthesia hypohidrosis, exercise intolerance, cataracts, corneal opacity, angiokeratoderma, proteinuria or renal tubular dysfunction, coronary artery disease, arrhythmia, gut dysfunction	Posterior circulation infarction more common, stroke phenotype includes haemorrhage and ischaemia

**Table 1-3: Single-gene disorders in children with arterial ischaemic stroke and cerebral arteriopathy.**

*Adapted from Munot et al. 2011a. AIS=arterial ischaemic stroke; AD=autosomal dominant; AR=autosomal recessive; PDA=patent ductus arteriosus; CADASIL=cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy; CARASIL=cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy; CSF=cerebrospinal fluid. IFN- $\alpha$ =interferon alpha; MOPD=microcephalic osteodysplastic primordial dwarfism type II; XLR=X-linked recessive; COL4A1 =collagen alpha-1(IV) gene; ABCC6= ATP-binding cassette, sub-family C, member 6; PCNT=pericentrin; SC SLC2A10/GLUT10 = Solute carrier family 2, facilitated glucose transporter number 10; CBS= cystathionine-beta-synthase; MHTFR= methylenetetrahydrofolate reductase; GLA=galactosidase alpha.*

## **1.5 Risk factors for arterial ischaemic stroke (AIS) recurrence**

Defining the risk of recurrent acute ischaemic stroke in individual patients is challenging given the diverse range of risk factors which may be present in different combinations in each case. In untreated children who suffer first AIS outside the neonatal period, the risk of recurrence is 10-25% overall but maybe as high as 92% in patients with SCD who receive no treatment (Barnes et al. 2004; Chabrier et al. 2000; Dobson et al. 2002; Fullerton, Wu, Sidney, & Johnston 2007; Ganesan et al. 2006). In the context of SCD the risk of recurrence appears to be highest in children with moyamoya (Dobson et al. 2002). There are a few long term follow up data for patients who develop AIS in the context of cardiac disease but both symptomatic and clinically silent recurrence have been described. In symptomatic and cryptogenic cases of childhood AIS recurrence is commoner if there are > 2 risk factors (Lanthier et al. 2000), and in those with arteriopathy particularly if it is progressive or if moyamoya collaterals are demonstrated angiographically and in those with low birth weight (Fullerton, Wu, Sidney, & Johnston 2007; Ganesan et al. 2006). Children with arterial disease or moyamoya have a risk of recurrence as high as 66% (Danchaivijitr et al. 2006; Fullerton, Wu, Sidney, & Johnston 2007; Ganesan et al. 2006). However the severity of recurrent symptoms with moyamoya varies considerably between patients with some having TIAs and others having fewer but devastating recurrent strokes (Danchaivijitr, Cox, Saunders, & Ganesan 2006; Fullerton, Wu, Sidney, & Johnston 2007; Ganesan et al. 2006).

Chabrier et al. demonstrated that patients with cardiac disease, moyamoya or systemic disease could have recurrence many years after the original stroke whereas those with TCA/FCA or dissection were more likely to have early recurrence (Chabrier et al. 2000). In

a UK based cohort genetic thrombophilias were significantly commoner in children with cryptogenic stroke who recurred (Ganesan et al. 2006). Furthermore, Braun et al. recently characterised the course of childhood unilateral intracranial arteriopathies in a cohort of 79 children and demonstrated a progressive arteriopathy, with increasing unilateral disease or bilateral involvement in 6% of the cases (Braun et al. 2009). This progressive arteriopathy was associated with recurrence as was arterial occlusion (Braun et al. 2009). In addition, Benseler et al. reported on 62 children with cerebral arteriopathies meeting the Calabrese criteria for a diagnosis of cPACNS and defined that those children at risk of progression had more often a clinical presentation of neurocognitive dysfunction, multifocal parenchymal lesions on MR imaging, and evidence of distal stenoses on angiography (Benseler et al. 2006). The authors proposed that these characteristic features at diagnosis could be used to predict later progression, and identify a distinct high at risk of progression cohort of children with cerebral arteriopathies that may benefit from immunosuppressive therapy (Benseler et al. 2006).

In summary, it is now clearly recognised that arterial disease is observed in the majority of children with AIS and that its presence and morphology are important predictors of subsequent course (Fullerton, Wu, Sidney, & Johnston 2007; Ganesan et al. 2006; Mackay et al. 2011). As discussed above some of these arteriopathies are due to defined causes such as arterial dissection, connective tissue disorders, inherited monogenic vascular disorders and SCD (Ganesan et al. 2003; Mackay et al. 2011). However, in up to 30% of children presenting with a first AIS, a focal arterial stenosis is identified but none of the classic underlying causes are discovered (Braun et al. 2009; Chabrier et al. 1998; Sebire et al. 2004).

To date, the underlying mechanisms for these unilateral, monophasic arteriopathies referred to as TCA and more recently as FCA are poorly understood despite the increased attendant risk of recurrent events of up to 20% in some case series of childhood arteriopathic stroke (Braun et al. 2009; Chabrier et al. 1998; Sebire et al. 2004).

## **1.6 CNS vasculopathy or vasculitis- semantic confusion and key area of controversy**

As the aetiology of cerebral arteriopathy presenting with AIS, specifically the role of infection and inflammation remains unclear, semantic confusion has arisen as to whether this cerebrovascular disease represents a true vasculitic process (Amlie-Lefond et al. 2009; Benseler et al. 2006; Braun et al. 2009; Eleftheriou & Ganesan 2009). Strong epidemiological evidence for an association between the proximal stenotic arterial disease in PVA and preceding VZV infection as well as the link of burden of recent upper respiratory tract infections to incident FCA, suggest that infectious agents, or the host response to infection, may be implicated in the pathogenesis of childhood cerebral arteriopathy and thus AIS (Amlie-Lefond et al. 2009; Askalan et al. 2001; Fullerton et al. 2011; Sebire, Meyer, & Chabrier 1999). In addition, some of the angiographic appearances of TCA/FCA and PVA, with beading and irregular multi-segmental involvement, as well as the radiological course with early progression and subsequent stabilization would be compatible with an inflammatory process (Aviv et al. 2006; Braun et al. 2009; Eleftheriou et al. 2010). Based on these arguments some authors (primarily rheumatologists) have suggested that these children have an isolated CNS vasculitis, referred to as primary angitis of the CNS in childhood

(cPACNS) (Benseler et al. 2006; Eleftheriou & Ganesan 2009; Hutchinson et al. 2010). Indeed this group of patients would fulfil the diagnostic criteria for cPACNS as proposed by Calabrese et al (Calabrese & Mallek 1988). The difficulty in distinguishing between cPACNS and presumed non-inflammatory monophasic TCA/FCA is related to the insensitivity of currently available investigations for cPACNS (Benseler et al. 2006; Eleftheriou & Ganesan 2009; Hutchinson et al. 2010). Essentially recognition and monitoring of cPACNS is heavily reliant upon imaging investigations that have no well-established or limited sensitivity and specificity (Aviv et al. 2006; Benseler et al. 2006; Eleftheriou et al. 2010; Eleftheriou & Ganesan 2009; Hutchinson et al. 2010). In addition, histology obtained by brain biopsy can provide definitive confirmation of vasculitis in a minority of cases, but unfortunately is invasive, lacks sensitivity (due to the patchy nature of the disease), and generally identifies parenchymal injury secondary to ischaemia which has resulted from vascular dysfunction rather than definitive evidence of vasculitis (Elbers et al. 2010; Elbers & Benseler 2008). Furthermore, in most series of cPACNS conventional circulating acute phase markers are lacking and specific circulating markers of cerebral vascular injury have yet to be defined (Benseler et al. 2006; Cellucci, Tyrrell, Sheikh, & Benseler 2011; Eleftheriou & Ganesan 2009; Hutchinson et al. 2010). As a consequence of these difficulties in distinguishing FCA from cPACNS, there are two parallel strands in the current literature, describing patients with very similar clinical and radiological features but to whom different diagnostic labels have been attached (cPACNS vs. FCA/PVA) and divergent management strategies adopted (Benseler et al. 2006; Braun et al. 2009; Eleftheriou & Ganesan 2009; Hutchinson et al. 2010). For example, Aviv et al describe vascular changes (typically unilateral stenosis of the proximal middle cerebral artery-MCA)

attributed to cPACNS (Aviv et al. 2006). In reports by Miravet et al and by Danchaivijitr et al very similar proximal lesions are interpreted as TCA/FCA (Danchaivijitr, Cox, Saunders, & Ganesan 2006; Miravet et al. 2007). Essentially both FCA and cPACNS manifest focal proximal occlusive disease of the intracranial arteries radiologically. However, FCA is generally monophasic, while PACNS typically has a recurrent course, possibly due to persistence of an aberrant inflammatory response, which is potentially modifiable by steroids and immunosuppressants (Benseler et al. 2006; Eleftheriou & Ganesan 2009; Hutchinson et al. 2010). The importance of making this distinction is more than semantic as immunosuppression is advocated for cPACNS but not for TCA/FCA which is thought to be a monophasic process. This controversy highlights the urgent need for studies into the pathogenetic mechanisms implicated in childhood cerebral arteriopathies and childhood AIS. Previous studies have proposed radiological and clinical predictors of arteriopathy progression and AIS recurrence but neither these nor any currently described biomarker, robustly distinguish between patients with a likely monophasic or a likely recurrent course thus complicating current management strategies (Benseler et al. 2006; Braun et al. 2011).

## **1.7 Treatment**

With the exception of SCD, the best available evidence for treatment of childhood ischaemic stroke is based on theory, extrapolation from adult studies, and cohort studies in children (Eleftheriou and Ganesan 2008). In the absence of acute treatments to limit brain injury, and absence of a population screening programme for risk factors for paediatric stroke, current approaches focus primarily on preventing stroke recurrence (Monagle et al 2004; Roach,



2008; Royal College of Physicians, 2004). Three separate treatment guidelines for childhood stroke have been published: the American College of Chest Physicians (ACCP) in the United States, the Royal College of Physicians (RCP) guidelines in the United Kingdom; and most recently the American Heart Association (AHA) Stroke Council scientific statement for management of stroke in children (Monagle et al 2004; Roach, 2008; Royal College of Physicians, 2004). Additionally the British Committee on Standards of Haematology (BCSH) have published evidence based recommendations on the therapy of cerebral sinovenous thrombosis (CSVST) in children (British Hematology society, 2007). All guidelines were created by expert groups that included paediatric neurologists and haematologists from multiple centres (Monagle et al 2004; Roach, 2008; Royal College of Physicians, 2004). They all acknowledge the lack of randomised control trials and therefore emphasize the limitations of the recommendations which are largely based on expert consensus (Monagle et al 2004; Roach, 2008; Royal College of Physicians, 2004).

Treatment guidance is broadly divided into therapy for the acute phase of AIS and prophylaxis to prevent stroke recurrence (Monagle et al 2004; Roach, 2008; Royal College of Physicians, 2004). The ACCP guidance supports early initiation of anti-coagulation with either low molecular weight heparin (LMWH) or unfractionated heparin for the first 5-7 days after AIS and until a cardiac source or dissection has been excluded (Monagle et al. 2004). Children are usually then changed to aspirin (3-5 mg/kg/d) or remain on anti-coagulation in cases due to cardiogenic embolism or dissection (Monagle et al. 2004). Similarly the AHA Stroke Council recommends that the administration of LMWH may be considered in children for up to 1 week after an ischaemic stroke pending further evaluation to determine

the cause of the stroke (Roach et al. 2008). In contrast, the RCP guideline recommends the initiation of aspirin at 5 mg/kg/day except in patients with the contraindications of intracranial haemorrhage or children with SCD (Royal College of Physicians, 2004). Early anti-coagulation is supported only in cases of proven arterial dissection and is considered in suspected cardiac thromboembolism (Royal College of Physicians, 2004). For children with arterial dissection or cardiac embolism, all three guidelines recommend anticoagulant therapy with warfarin or LMWH for 3–6 months (Monagle et al 2004; Roach, 2008; Royal College of Physicians, 2004). Even though clinical trials on adult patients with AIS have shown reduced morbidity and mortality in patients treated with intravenous tissue plasminogen activator (tPA) within hours from stroke onset, the theoretical risks and lack of evidence in children resulted in neither the RCP, the AHA Stroke Council or the ACCP guidelines recommending the use of thrombolytics in paediatric stroke (Monagle et al 2004; Roach, 2008; Royal College of Physicians, 2004). These current treatment guidelines also differ in their recommendations for long-term treatment of children after AIS, with the ACCP guidance recommending maintenance aspirin in all patients and the others only in children with vasculopathy (Monagle et al 2004; Roach, 2008; Royal College of Physicians, 2004). Management of stroke in SCD includes initial and maintenance blood transfusions (see section on SCD 1.2.4) (Adams et al. 1998; Monagle et al 2004; Roach, 2008; Royal College of Physicians, 2004). Endovascular therapies are increasingly used particularly in moyamoya arteriopathy (Khan et al. 2003; Scott et al. 2004). Treatment of cPACNS is summarised in 1.2.2.3. Clinical trials to address areas of divergent opinion and improve the evidence base for rational treatment of childhood stroke in the near future are urgently needed. The three clinical guidelines are summarised in table 1-4.

<b>Acute Childhood AIS</b>	<b>RCP guidelines (2004)</b>	<b>ACCP guidelines (Monagle et al. 2004)</b>	<b>AHA Stroke Council (Roach et al. 2008)</b>
General	Aspirin 5mg/kg/day	UFH or LMWH for 5 to 7 days and until cardioembolic and dissection have been excluded as causes	The administration of LMWH or UFH may be considered in children for up to 1 week after an ischaemic stroke pending further evaluation to determine the cause of the stroke.
Sickle cell disease	Exchange transfusion to HbS<30%	Intravenous hydration and exchange transfusion to HbS<30%	Intravenous hydration exchange transfusion to reduce HbS to <30% total haemoglobin
Thrombolysis	Not recommended	Not recommended	Not recommended
<b>Maintenance therapy in childhood AIS</b>			
General	Aspirin 5mg/kg/day	For all children with AIS treat with ASA 2–5 mg/kg/day after anticoagulation therapy has been stopped	
Dissection	Consider anti-coagulation until evidence of vessel healing or up to 6 months	After 5–7 days UFH or LMWH, treat with LMWH or warfarin for 3–6 months	For extracranial dissection LMWH or warfarin for 3 to 6 months or an antiplatelet agent should be considered. Extend therapy beyond 6 months for individuals who develop recurrent symptoms or when there is radiographic evidence of a residual abnormality of the dissected artery. Anti-coagulation not recommended for intracranial dissection.
Cardioembolic	Consider anticoagulation	After 5–7 days UFH or LMWH, treat with LMWH or warfarin for 3–6 months	Cardioembolic unrelated to PFO after initial therapy with UFH or

			LMWH, treat with LMWH or warfarin for 3-6 months. For children with a suspected cardiac embolism unrelated to a PFO with a lower or unknown risk of stroke, it is reasonable to begin aspirin and to continue it for at least 1 year.
Vasculopathy	Aspirin 1-3 mg/kg/day	Aspirin 2-5 mg/kg/day after anticoagulation therapy has been stopped	Aspirin 3-5mg/kg/day
Sickle cell disease	Blood transfusion every 3-6 weeks Long-term transfusion programme to HbS 30% After 3 years aim for HbS 50% If no transfusion, hydroxyurea Consider bone-marrow transplant	Long-term transfusion programme	Long term transfusion programme. Hydroxyurea may be considered in patients who cannot continue on long-term transfusion. Consider bone marrow transplant
Recurrent stroke on aspirin	Consider anticoagulation	Not addressed	Not addressed

**Table 1-4: Treatment guidelines for paediatric stroke-antithrombotic therapy.**

*ACCP=American College of Clinical Pharmacy; RCP=Royal College of Physicians; AHA=American Heart Association; AIS=arterial ischaemic stroke; INR=international normalised ratio; LMWH=low molecular weight heparin; UFH=Unfractionated heparin. Child defined as 28 days to 18 years (ACCP), 1 month to 16 years (RCP), 28 days to 16 years (AHA Stroke Council).*

## 1.8 Outcome

The reported mortality after AIS in childhood varies widely between different series from 3 to 23% (Ganesan et al. 2000; Gordon, Ganesan, Towell, & Kirkham 2002; MacGregor, Curtis, & Mayank 2000; Neuner et al. 2011). Childhood AIS also has the potential for life-long morbidity (Ganesan et al. 2000; Gordon, Ganesan, Towell, & Kirkham 2002; MacGregor, Curtis, & Mayank 2000; Neuner et al. 2011). The primary outcome of childhood AIS based on Pediatric Stroke Outcome Measure (PSOM) assessments in a recent study by DeVeber et al. suggested moderate to severe deficits in the majority of patients (MacGregor, Curtis, & Mayank 2000). Multivariate analysis showed that AIS, associated neurologic disorders, and presence of rehabilitation therapy were independent predictors of poor outcome ( $P < 0.02$ ) (MacGregor, Curtis, & Mayank 2000). In addition, Neuner et al. showed in a recent study of 133 survivors of childhood AIS that 65% exhibited at least 1 neurologic disability years after incident stroke (Neuner et al. 2011). Paediatric stroke survivors also reported lower overall well-being compared with healthy controls (Neuner et al. 2011). Lastly, Gordon et al. recently reported on the functional consequences of childhood AIS in terms of activity limitation showing that this was evident in the domains of education, self-care, and motor skills (Gordon, Ganesan, Towell, & Kirkham 2002). There was in addition a clear relationship between radiologically apparent extent of brain damage, degree of impairment, and functional outcome (Gordon, Ganesan, Towell, & Kirkham 2002).

## 1.9 Conclusion

In summary AIS is an increasingly recognised childhood neurological disorder resulting in long term morbidity in two thirds of survivors and recurrence in a fifth (Ganesan et al. 2000; Gordon, Ganesan, Towell, & Kirkham 2002; MacGregor, Curtis, & Mayank 2000; Neuner et al. 2011). AIS is usually secondary to cerebral arteriopathy, the morphology and evolution of which is highly predictive of recurrence (Fullerton, Wu, Sidney, & Johnston 2007; Ganesan, Prengler, Wade, & Kirkham 2006; Mackay et al. 2011). Preceding VZV infection has been identified as a predisposing factor to cerebral arteriopathies while minor upper respiratory tract infections have been reported as an independent risk factor for focal cerebral arteriopathies, providing clues that viral infection and/or a secondary inflammatory response may be involved in the pathophysiology of these conditions (Amlie-Lefond et al. 2009; Askalan et al. 2001; Sebire, Meyer, & Chabrier 1999). In addition, inherited or acquired thrombophilias likely interact in concert with other identified risk factors mainly cerebral arteriopathy to initiate and/or propagate thromboembolism in childhood AIS patients (Bernard et al. 2010; Kenet et al. 2010). Several clinical studies have attempted to identify a “high” risk profile of children with AIS and cerebral arteriopathy that are more at risk of a second stroke but to date no currently described biomarker robustly distinguishes between those children with a monophasic disease course and those with recurrence (Benseler et al. 2006; Braun et al. 2009; Cellucci, Tyrrell, Sheikh, & Benseler 2011). Progression of cerebral arteriopathy and AIS recurrence may be related to persistence of cerebral arterial injury and chronic sub-clinical endothelial activation conferring an increased thrombotic risk. If this is the case there may be a role for novel interventions, such as anti-inflammatory agents, in

secondary stroke prevention. As these treatments have potential side effects identification of biomarkers of vascular injury and prothrombotic risk associated with AIS recurrence, could allow us to select those children more at risk of AIS recurrence who may benefit from immunosuppression whilst avoiding unnecessary cytotoxic treatment in those with a more benign course. Recently described methods for detecting endothelial cell components in peripheral blood may allow early detection of endothelial injury indicative of persistence and/or propagation of cerebral vascular inflammation in these children with AIS recurrence and would be of considerable clinical interest (Hirschi et al. 2008; Morel et al. 2006; Woywodt et al. 2002).

## **1.10 Non-invasive detection of endothelial injury and repair capacity**

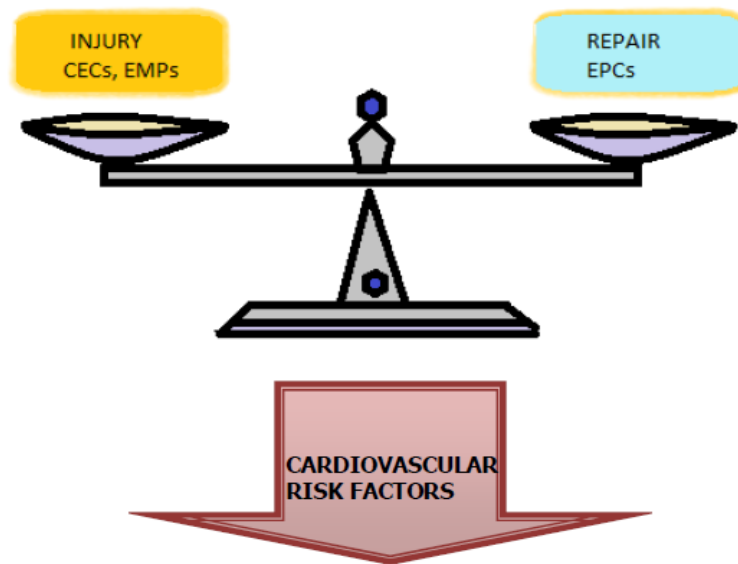
### ***1.10.1 Dynamics between endothelial injury and repair***

Once considered to serve no other purpose than that of a physical barrier between blood and tissue the multifunctional nature of the endothelium was discovered in the later part of the 20<sup>th</sup> century (Sabatier et al. 2009). The endothelium is a highly dynamic tissue in equilibrium with the circulating compartment and composed of various sub-populations offering important opportunities for a non-invasive exploration (Sabatier et al. 2009). It is now well recognized that the balance between vascular injury and repair is critical for the maintenance of vessel integrity (Sabatier et al. 2009). In addition, disruption of endothelial integrity and altered endothelial function represent an important mechanism in the development of cardiovascular disorders (CVD) including cerebrovascular disease (Blann et al. 2002; Felmeden et al. 2003; Sabatier et al. 2009). Chronic exposure to cardiovascular risk factors (CRF) alters the regulatory function of the endothelium that progresses from a quiescent state to activation, apoptosis and death (Bazzoni and Dejana 2004; Sabatier et al. 2009; Varani and Ward 1994). As a result the endothelium not only displays altered functions, but also loses its integrity (Sabatier et al. 2009). Microparticles (membrane vesicles <1 µm in size and rich in phosphatidylserine) released from activated or apoptotic endothelial cells and whole endothelial cells detached from injured vessels (circulating endothelial cells; CEC) constitute a fundamental feature of these injurious responses affecting the vessel wall (Blann et al. 2005; Dignat-George and Boulanger 2011; Piccin et al. 2007; Woywodt et al. 2003). In response to injury, regenerative mechanisms are activated to restore endothelium

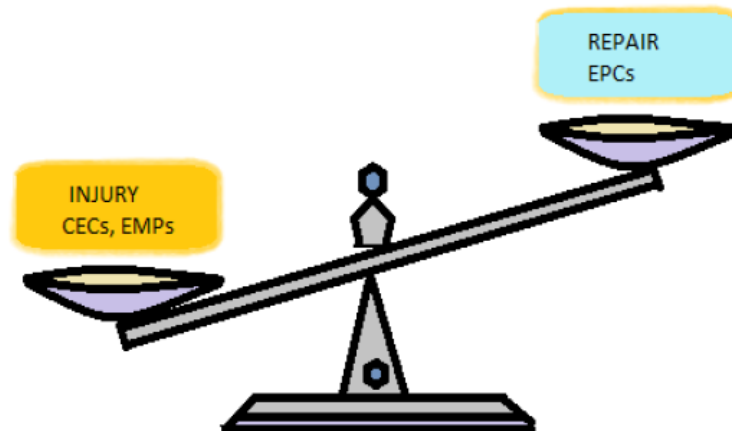


integrity (Hirschi, Ingram, & Yoder 2008; Sabatier et al. 2009; Yoder et al. 2007). In the past, endothelial repair was considered to solely involve adjacent endothelial cells able to replicate locally and replace the lost cells (Hirschi, Ingram, & Yoder 2008; Rabelink et al. 2010; Richardson and Yoder 2011). Since the original study by Asahara et al. it has become obvious that the recruitment of endothelial progenitor cells (EPC) represents an additional mechanism for vascular repair (Asahara et al. 1997; Hill et al. 2003; Hirschi, Ingram, & Yoder 2008; Werner et al. 2005; Yoder, et al. 2007). These stem cells are mobilized from the bone marrow and are able to differentiate into mature cells, restoring endothelial integrity at sites of vascular injury (Asahara et al. 1997). This spectrum of endothelial responses can be thus considered in a dynamic triad ‘activation/injury/repair’ which has critically transformed our understanding of endothelial biology (figure 1-2) (Sabatier et al. 2009). Whereas the determination of CEC and endothelial derived MP levels have raised considerable interest to appreciate the status of activated/ damaged endothelium, EPC levels have been used to evaluate the endogenous repair potential (Erdbuegger et al. 2008; Hill et al. 2003; Sabatier et al. 2009; Werner et al. 2005; Woywodt, 2003). Combined measurement of these indices offers a non-invasive and original way to estimate vascular competence in individual patients at different time points (Clarke et al. 2010; Sabatier et al. 2009). We can therefore begin to develop biomarker strategies to identify patients with high vascular risk such as perhaps children with AIS at risk of recurrence, and potentially monitor their response to treatment by assessing the balance between injury and repair markers as an index of overall endothelial integrity.

## ENDOTHELIAL INTEGRITY



## DISRUPTED ENDOTHELIAL INTEGRITY



**Figure 1-2: Dynamics of endothelial injury and repair.**

*Disruption of endothelial integrity is associated with a spectrum of responses including detachment of whole endothelial cells (CEC) and release of endothelial microparticles (EMP). In response to injury, endothelial progenitors cells (EPC), recruited from the bone marrow play a role in restoring endothelial integrity.*

## **1.11 Hypothesis and aims of this thesis**

### **1.11.1 Hypothesis**

This thesis explored the following hypothesis:

In some children with AIS and cerebral arteriopathy, there is persistent sub-clinical cerebral arterial endothelial activation and injury long after the inciting insult has disappeared. Simultaneously, abnormalities in repair of chronic endothelial injury due to altered endothelial progenitor cell responses impair recovery from childhood AIS. This unfavorable balance of endothelial injury and repair contributes to AIS recurrence and further neurological events.

### **1.11.2 Aims:**

The aims of this thesis were to:

- 1) Investigate whether biomarkers of endothelial injury can be detected in the peripheral circulation of children with AIS and occlusive cerebral arteriopathy (with the exclusion of children with sickle cell disease or other systemic vascular disorders) and whether these indices differentiate children with recurrent AIS from those with a monophasic disease course.
- 2) Evaluate novel biomarkers of thrombotic propensity in children with AIS and cerebral arteriopathy and explore whether these are associated with recurrent AIS.
- 3) Assess whether AIS recurrence is associated in part with altered endothelial progenitor cell responses impairing recovery from childhood AIS.

## **2 Methods and materials**

### **2.1 Introduction**

This section contains predominantly materials and methods that are used in more than one chapter. Methods applicable to one chapter only or those that have been developed or adapted specifically for this study will be discussed in detail in the relevant chapter.

### **2.2 Subjects**

#### ***2.2.1 Study design and patient population***

Cross-sectional study of children aged >28 days old with arterial ischaemic stroke (AIS) and imaging evidence of cerebral/cervical arteriopathy presenting to Great Ormond Street Hospital (GOSH) from October 2007 to January 2012. GOSH is the tertiary paediatric neurology center serving north London, United Kingdom. Patients were identified by a search of the paediatric neurology service outpatient and inpatient database.

AIS was defined as an acute focal neurological deficit attributable to cerebral infarction in a corresponding arterial distribution. Cerebral/cervical arteriopathy was defined as focal or segmental stenosis or occlusion, with regular or irregular abnormalities of the arterial wall (Braun et al. 2009) and categorized according to the consensus definitions published by Sebire et al. (Sebire et al. 2004). However, the category of transient cerebral arteriopathy (TCA) in this classification has more recently been superseded by the term focal cerebral

arteriopathy (FCA) which was used in this study (Amlie-Lefond et al. 2009). The Childhood AIS Standardized Classification And Diagnostic Evaluation (CASCADE) categorisation is also provided for comparison (see appendix 1 for details) (Bernard et al. 2012). This is a comprehensive consensus-based classification system for childhood AIS recently proposed and validated by the International Paediatric Stroke Study (IPSS) group (Bernard et al. 2012).

Exclusion criteria were: patients with other systemic conditions that can cause endothelial injury (sickle cell disease, systemic vasculitis, systemic lupus erythematosus or other autoimmune connective tissue disease); those with known cardiac disease; those with histopathologically confirmed cerebral vasculitis; syndromic moyamoya and those with other potentially confounding syndromic diagnoses. Patients with arterial occlusion but no other arterial wall changes were also excluded from the study as these radiological appearances could result from cardioembolic or paradoxical embolism.

In addition to the cross sectional study, children with a new presentation of AIS and cervical/cerebral arteriopathy were identified at presentation to the neurology services at GOSH and evaluated prospectively over a 12 month period.

### ***2.2.2 Healthy controls and disease controls***

Blood samples were obtained from healthy controls comprised of an unselected sequential group of children attending the surgical ward at GOSH between October 2007 and January

2012 and undergoing minor surgical procedures (such as hernia repair; pre-operative blood samples obtained) with no identifiable medical history of acute or chronic illnesses or syndromic diagnosis, and in particular no evidence of intercurrent infection. Healthy adult volunteers were staff from within the Infection and Immunity Theme at the Institute of Child Health, University College London (UCL).

A disease control group was included comprising of children with cerebral arteriovenous malformations (AVM) as these patients have non-inflammatory vascular pathologies.

Informed consent was obtained from all parents/guardians and participants/child healthy controls with local ethics approval (COREC 07/Q0508/58).

### ***2.2.3 Clinical, laboratory and radiological data***

All children with AIS routinely underwent magnetic resonance (MR) imaging and MR angiography within 48 hours of presentation for confirmation of the diagnosis and identification of possible cerebral/cervical arteriopathy.

MRI and MRA were performed on a 1.5-Tesla MR scanner using a standard imaging protocol, including T2-weighted turbo spin-echo imaging in the axial plane, fluid-attenuated inversion recovery (FLAIR) sequence in the coronal plane, T1-weighted spin-echo imaging in the sagittal plane, and three-dimensional short-echo time-of-flight MRA of the circle of Willis. In the under 2 age group, the axial T2-weighted imaging was replaced by dual echo stimulated echo (deSTIR) imaging sequence and the coronal FLAIR sequence was not

acquired. Catheter cerebral arteriography (CA) was performed on a biplane angiographic system with selective catheterization and contrast injections into both internal carotid arteries (ICA) and into at least one vertebral artery (VA). CA was performed at the clinician's discretion, as this may provide adjunctive yield for detection of arteriopathy (Eleftheriou et al. 2010). Blood was drawn before CA was performed to exclude procedure-related endothelial injury. Cerebral/cervical arteriopathy was identified on MRA, and/or CA where available, and categorised using currently recommended definitions. Proximal, distal, or whole segment involvement was identified. Distal involvement was defined as involvement beyond the M1 or A1 segments. Presence of collateral vessels was determined. Patients were re-imaged with MRI/MRA at 6 and 12 months following index AIS and annually thereafter for clinical monitoring.

All children underwent comprehensive investigation for AIS risk factors (appendix 2). These included: erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP); clotting profile and thrombophilia screen specifically testing for protein C, protein S, antithrombin III, plasminogen, lupus anti-coagulant and antiphospholipid antibodies, activated protein C resistance ratio, Factor V Leiden, thermolabile variant of methylenetetrahydrofolate reductase (t-MTHFR) and prothrombin 20210 gene mutations; plasma amino acids, homocysteine, urate, ammonia, lactate; and lipoprotein(a) levels. Intercurrent infection as a potential important confounding factor was excluded based on detailed history and clinical examination. Treatment was based on current international management guidelines (Monagle et al 2004; Roach, 2008; Royal College of Physicians, 2004). Two treatment guidelines for childhood ischaemic stroke have been published and are those of the Royal

College of Physicians in the UK and more recently the American Heart Association Stroke Council (Roach, 2008; Royal College of Physicians, 2004). In addition, the American College of Chest Physicians provides guidance on the use of anticoagulants and antithrombotic therapy in childhood stroke (Monagle et al. 2004). Supportive care and neuroprotective strategies are important considerations in children with ischaemic stroke (Eleftheriou & Ganesan 2008; Roach et al. 2008). Antithrombotic strategies are advocated; the choice of agent varies according to the underlying risk factors (Monagle et al 2004; Roach, 2008; Royal College of Physicians, 2004). Due to the theoretical risks and lack of evidence thrombolytic treatment is not generally recommended in paediatric stroke (Monagle et al 2004; Roach, 2008; Royal College of Physicians, 2004). Lastly, specific pathologies may require targeted management, for example surgical revascularization for moyamoya (Eleftheriou & Ganesan 2008; Roach et al. 2008).

#### ***2.2.4 Patient groups***

Patients were categorised into two groups according to AIS recurrence. AIS recurrence was defined as a sudden onset of new neurological deficit with radiological evidence of re-infarction and identified at re-presentation to our institution. Of note since the mode of presentation of AIS in children is often fluctuating and recurring, recurrent AIS was defined only when the new clinical event occurred > 1 week after presentation (Braun et al. 2009). Where clinical recurrence did occur, children underwent additional MRI/MRA imaging to identify new areas of arterial territory infarction and/or progression of arteriopathy, defined as progression of previously identified arterial disease (more extensive segment involved,



more severe occlusive disease) and/or involvement of previously unaffected vessels and/or new arterial occlusion in the presence of arterial wall changes.

## **2.3 Materials**

### ***2.3.1 Reagents for blood collection***

After blood was collected it was aliquoted into one or more of the following collection bottles depending on use: 1.4ml of 3.2% trisodium citrate (Sartstedt), 5 ml Ethylenediaminetetraacetic acid (EDTA; International specific supplies), 5 ml polypropelene containing no anticoagulant or sterile 20 ml universal bottles containing 40µl of preservative free heparin (monoparin, CP pharmaceuticals Ltd, 1000 U/ml).

### ***2.3.2 Fluorochrome conjugated antibodies for flow cytometry***

The fluorochrome antibodies used are listed in table 2-1. Antibodies were diluted with 0.01 M phosphate buffered saline with 0.1% sodium azide for staining of peripheral blood mononuclear cells (PBMC) and Human Umbilical Vein Endothelial Cells (HUVEC). For staining of microparticles (MPs) antibodies were diluted with sterile filtered RPMI 1640 supplemented with 5% heat inactivated FCS and 0.1% sodium azide. All antibodies were titrated by plotting a dilution curve against median fluorescence index, the relevant dilution for each antibody corresponding to the shoulder of the curve. Each antibody was also checked against an appropriate isotype-control antibody with the same protein concentration, as per manufacturers' recommendation. For peripheral blood mononuclear cells (PBMC)

staining FC receptor blocking reagent (Miltenyi biotech) was used to further reduce nonspecific binding.

<b>Specificity</b>	<b>Isotype</b>	<b>Conjugate</b>	<b>Company</b>	<b>Clone</b>	<b>Dilution</b>
CD41	Mouse IgG1κ	TRICOLOR	Caltag laboratories	Clone VIPL3	1/50
CD42a	Mouse IgG1κ	PERCP	BD PharMingen	Beb1	1/50
activation epitope- CD11b	Mouse IgG1, κ	PE	Biolegend	CBRM1/5,	1/50
CD142	Mouse IgG1κ	FITC	American Diagnostica	clone VD8,	1/50
CD62e	Mouse IgG1κ	PE	BD PharMingen	clone 68- 5H11	1/50
CD54	Mouse IgG1κ	PE	BD PharMingen	LB-2	1/50
CD106	Mouse IgG1κ	PE	BD PharMingen	51-10C9	1/50
CD31	Mouse IgG1κ	PE	BD PharMingen	L133.1	1/50
CD62P	Mouse IgG1κ	PE	BD PharMingen	AC1.2	1/50
CD66b	Mouse IgG1κ	PE	BD PharMingen	G10F5	1/50
CD14	Mouse IgG1κ	PE-Cy5	AbD Serotec	61D3	1/50
CD162	Mouse IgG1κ	PE	BD Pharmingen	KPL-1	1/50
CD133	Mouse IgG1	PE	Miltenyi Biotec	clone AC133	1/10

CD34	Mouse IgG1	PERCP	BD Pharmingen	clone 8G12	1/10
VEGFR2	Mouse IgG1	Biotin	Sigma Aldrich	clone KDR2	1/10
CD144	Polyclonal IgG	FITC	AbD Serotec	n/a	1/10
Streptavidin	n/a	FITC	AbD Serotec	n/a	1/150

*Table 2-1: Fluorochrome conjugated antibodies/reagents for flow cytometry.*

### **2.3.3 Tissue culture media**

The following tissue culture media were used: RPMI-1640 culture medium containing 2 mM L-glutamine (Life Technologies, Gibco BRL) and supplemented with antibiotics (100 IU/ml penicillin; 10 µg/ml streptomycin, and 25 ng/ml amphotericin B); foetal calf serum (heat inactivated at 56° C for 60 minutes to destroy complement); endothelial Cell Growth Medium 2 (EGM-2;Promocell) comprising of basal endothelial growth medium supplemented with 2% foetal calf serum (FCS), 5ng/ml of epidermal growth factor, 10 ng/ml of basic fibroblast growth factor, 10 ng/ml of insulin growth factor, 0.5 ng/ml of Vascular Endothelial Growth Factor (VEGF), 1 µg/ml of ascorbic acid, 0.2 µg/ml of hydrocortisone and 90 µg/ml of heparin as per manufacturer's instructions.

### **2.3.4 Cytokine and protein reagents**

The following cytokine was used: recombinant TNF- $\alpha$  (100 ng/ml, Sigma Aldrich). The following peptides were used formyl-methyl-leucyl-phenylalanin (fMLP; 1 $\mu$ M from Sigma Aldrich), lipopolysaccharide (LPS) derived from *E. Coli*, O55:B5 (5  $\mu$ g/ml, Sigma). The following fluorochrome conjugated proteins were used: annexin V (unconjugated, FITC, PE or PE-Cy5 labeled; 1:50 dilution, BD); Ulex europaeus I agglutinin (UEA-I FITC conjugated, 10 mg/ml, Sigma Aldrich), Streptavidin (FITC, 1:150 dilution, Serotec). Trypsin-EDTA (Promo cell) and trypsin neutralizing solution /TNS (0.05 % trypsin inhibitor, 0.1 % BSA; Promo cell) were used for detachment of human umbilical vein endothelial cells (HUVEC) from culture. Corn trypsin inhibitor (30  $\mu$ g/ml; Sigma) was used to inhibit in vitro contact activation of the coagulation cascade in the thrombin generation assay (TGA).

### ***2.3.5 Bead based extraction reagents***

DYNAL bead technology (Invitrogen) was used for isolation of circulating endothelial cells (CECs): Dynabead Pan mouse IgG were labeled with an unconjugated IgG1 antibody to CD146, clone s-endo1 (Biocytex). The Dynal MCP-L magnet which holds 1-8 ml tubes was also used.

### ***2.3.6 ELISA kits***

Levels of von Willebrand factor were determined using a commercially available kit (American Diagnostica) as per manufacturer recommendation.

### **2.3.7 Other reagents**

Other reagents included:

- For isolation of peripheral blood mononuclear cells (PBMC) the density centrifugation separation reagent Lymphoprep<sup>TM</sup> (Axis-Shield) was used.
- Polymorphprep<sup>TM</sup> (Axis Shield) sterile and endotoxin tested solution for the isolation of polymorphonuclear cells containing 13.8% (w/v) sodium diatrizoate and 8.0% (w/v) polysaccharide was used for the isolation of peripheral blood neutrophils.
- Tyrode's buffer (Sigma Aldrich) containing 0.1% glucose, 0.1% BSA, 137.5 mmol/L NaCl, 12 mmol/L NaHCO<sub>3</sub>, 2.6 mmol/L KCl was used for the isolation of peripheral blood platelets.
- Annexin V binding buffer (BD Pharmingen) 10 x concentrate composed of 0.2 µM sterile filtered 0.1M Hepes (pH 7.4), 1.4M NaCl, and 25 mM CaCl<sub>2</sub> solution. Prior to staining, an appropriate quantity of a working solution was made by diluting the concentrate 1:10 with distilled water.
- Cell fix buffer (BD Biosciences): filtered distilled water plus 10% formaldehyde.
- Low-density lipoprotein from human plasma, acetylated, DiI complex (Di I Ac-LDL; Invitrogen).
- BD Matrigel Basement Membrane Matrix (BD Biosciences) is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in extracellular matrix proteins. Its major component is

laminin, followed by collagen IV, heparan sulfate proteoglycans, entactin/nidogen. BD Matrigel Basement Membrane Matrix also contains TGF-beta, epidermal growth factor, insulin-like growth factor, fibroblast growth factor, tissue plasminogen activator, and other growth factors which occur naturally in the EHS tumor.

- Calcium-fluorogenic substrate (0.5 mM/L of Z-Gly-Gly-Arg-7-Amino-4-Methylcoumarin and 7.5 mM/L of calcium final reagent concentrations, Pathway Diagnostics) for the thrombin generation assay.
- Human tissue factor pro-coagulant activity was measured using a commercially available kit (American Diagnostica) as per manufacturer recommendation.
- The following latex beads were used to investigate forward and side scatter (FSC/SSC) flow cytometric characteristics of particles: 1 $\mu$  and 3  $\mu$ m beads (Sigma).

## **2.4 Preparation of blood and tissue samples**

All human blood and tissue samples were collected with full ethical approval and informed consent (COREC 07/Q0508/58).

### ***2.4.1 Human umbilical vein endothelial cells (HUVEC) culture***

Commercially available HUVEC bought frozen from Promo Cell were resuspended in 10ml of EGM2 (Promocell) supplemented with 2% Fetal Calf Serum (FCS) and growth factors as

supplied by the manufacturer, and were then transferred to 25cm<sup>2</sup> flask. These were incubated at 37°C and 5% CO<sub>2</sub>. Cells were washed in fresh culture media the following day and inspected under phase contrast microscopy. HUVEC could be identified as small clusters of oval adherent cells. Cultures were inspected each day for growth. When cells were approaching confluence (which usually occurred after 72 hours culture), they were sub-cultured. For experimental purposes, fully confluent HUVEC at passages 2–3 were pre-incubated overnight with 0.5% FCS in EGM-2 prior to addition of factors and other experimental conditions described in the relevant sections.

#### ***2.4.2 HUVEC culture passage***

HUVEC that were near-confluent were washed three times in warmed PBS to remove non-adherent cells and protein in FCS. Cells were then washed once in 1.5 ml Trypsin-EDTA solution (per 25-cm<sup>2</sup> flask) which was then removed and cells were inspected under phase-contrast microscopy, and when rounding and becoming dislodged (usually after 30 seconds), the flask was tapped against the bench top to aid removal and a further 1.5-ml trypsin-neutralising solution added. The detached cells were quickly resuspended in warmed HUVEC culture medium and transferred to a 50-ml sterile conical tube. These were then seeded into 6 or 12 well flat-bottom tissue culture plates. Tissue culture plates were then incubated at 37°C in 5% CO<sub>2</sub> until cells were treated with trypsin again prior to use in various assay described in relevant sections. Of note as HUVEC morphology and cell adhesion molecule expression in response to inflammatory stimuli may alter after serial passage, the cells used throughout this study were always first to third sub-culture passage.

### ***2.4.3 Isolation of peripheral blood mononuclear cells (PBMC)***

Blood samples for PBMC isolation were collected into sterile universals containing 40 units of preservative free heparin (CP Pharmaceuticals LTD, Wrexham) and processed within 2 hours. Blood was diluted with an equal volume of culture medium containing RPMI 1640 medium (Invitrogen, Paisley, UK) and 100U/ml Penicillin/100 $\mu$ g/ml Streptomycin (Invitrogen). Blood-RPMI mix was layered onto 10 ml of Lymphoprep TM (Axis Shield). These samples were then centrifuged at room temperature at 800g for 25 minutes with no brake. The resulting PBMC interface was removed and an equal volume of culture medium was added and centrifuged at 500g for 10 minutes. The resulting pellet was resuspended in 40 ml of culture medium by gentle flicking and the PBMC counted as described in 3.4.4. Cells were centrifuged at 500g for 10 minutes and then resuspended in appropriate volume of freezing medium or EGM-2 (Promo-cell) before culturing.

### ***2.4.4 Counting viable cells***

Viable cells were quantified as follows: 10  $\mu$ l of cell suspension was mixed with 10  $\mu$ l of 0.4% trypan blue (Sigma). Half of the mix was placed on Neubauer counting chamber and cells enumerated by light microscopy. Unstained live cells were counted in the specified 25-box field and the total number calculated. Number of cells in 25-box field x 2 (dilution factor) x  $10^4$ =total number of viable cells per ml. Cell preparations were typically composed of > 97% viable cells.



#### ***2.4.5 Freezing cells***

Processed cells were cryopreserved in liquid nitrogen for long term storage. After counting cells were centrifuged and re-suspended at a concentration of  $2 \times 10^6$ /ml in freezing medium (FCS supplemented with 10% v/v dimethyl sulphoxide from Sigma). 1 ml aliquots were transferred into individual cryovials which were placed into a freezing pot with isopropanol coolant for 24 hours at  $-80^{\circ}\text{C}$  (allowing slow cooling) over night before transfer to liquid nitrogen storage.

#### ***2.4.6 Recovering of frozen cells***

Cryo-preserved cells were removed from liquid nitrogen and rapidly thawed in a  $37^{\circ}\text{C}$  water bath. Cells were then transferred to 2 ml of FCS in a drop wise fashion. Culture medium was added prior to centrifugation (500g for 10 minutes) before being counted.

#### ***2.4.7 Isolation of peripheral blood monocytes***

PBMCs isolated as above were resuspended at  $2 \times 10^6$  in RPMI 1640 medium (Invitrogen), plated into a 6 well plate and incubated for 3 hours to allow adherence of monocytes to the plate. Non adherent cells were removed and the adherent cell layer washed twice, each time with 10 ml RPMI (to remove any residual nonadherent cells) and then replaced with 10 ml fresh, serum-free RPMI 1640 medium supplemented with 100U/ml Penicillin/100 $\mu\text{g}$ /ml Streptomycin (Invitrogen). Adherent monocytes were removed by gently scraping with a

plastic cell scraper. Cells were then transferred to a 15-ml conical tube and centrifuged for 10 min at  $300 \times g$ , room in room temperature prior to resuspension in appropriate volume of culture medium and used in experimental conditions as discussed in the relevant chapters.

#### ***2.4.8 Isolation of peripheral blood neutrophils***

Neutrophils were isolated using Polymorphprep<sup>TM</sup> (Axis Shield) from 5-20 mls of blood obtained from adult volunteers and anti-coagulated with preservative-free heparin (Monoparin, CP Pharmaceuticals Ltd, 1000 U/ml). Briefly blood was carefully layered onto equal volume of Polymorphprep TM (Axis Shield). These samples were then centrifuged at 500g for 35 minutes with no brake. The resulting polymorphonuclear cell interface was harvested and an equal volume of culture medium containing RPMI 1640 medium (Invitrogen, Paisley, UK) diluted with water (1:1) was added and centrifuged at 400g for 10 minutes. The resulting pellet was re-suspended in 40 ml of culture medium by gentle flicking and the cells counted, washed twice and finally re-suspended in an appropriate volume of culture medium before used in experimental conditions as discussed in the relevant chapters.

#### ***2.4.9 Isolation of peripheral blood platelets***

Platelet-rich plasma was prepared by centrifugation of citrated blood at  $1000 \times g$  for 3 min. Immediately after 0.5 nM prostacyclin (Sigma Aldrich) was added to prevent in vitro platelet aggregation. To obtain a platelet pellet, platelet rich plasma was then centrifuged at at 5000g

for 15 minutes and the platelets were gently resuspended in Tyrode's buffer (Sigma Aldrich) and washed twice before stimulation in various experimental conditions as described in the relevant chapters.

#### **2.4.10 Preparation of platelet poor plasma**

Blood from patients/child controls and adult healthy volunteers was collected in 3.2% buffered citrate and centrifuged at 5000 g for 5 min twice to obtain platelet-poor plasma (PPP). PPP samples were divided into aliquots and stored at  $-80^{\circ}\text{C}$  for future batch testing. PPP samples were used for detection of microparticles and ELISA for von Willebrand factor.

## **2.5 Statistical analysis**

A number of parametric and non-parametric statistical analyses were used in this thesis. Description of tests and their application is given in the relevant chapters. Parametric tests used were student's t-test and paired t-tests. Non parametric tests used were: the Kruskal-Wallis test, Mann-Whitney U test, the Wilcoxon signed rank test and Spearman's rank correlation coefficient. Receiver operator characteristic (ROC) curves, sensitivity, specificity, positive and negative predictive values, and likelihood ratios were calculated to examine the diagnostic characteristics of indices described. ROC curve were reported as area under the curve (AUC) and 95% confidence intervals (CI). Levels of agreement for CEC counts between different investigators were summarised using Bland–Altman plots. Bland-

Altman plots were in addition performed when comparison of methodology was required. Results were expressed as bias SD and 95% CI of limit of agreement. P-values of less than 0.05 (two sided) were regarded as significant. Statistical analysis was performed using SPSS versions 16 and 17. Data are presented using GraphPad Prism (San Diego, CA).

# 3 Circulating endothelial cells in children with arterial ischaemic stroke

## 3.1 Summary

**Background:** Arterial ischaemic stroke (AIS) is commonly associated with abnormalities (arteriopathies) of the cervical or intracranial circulation the morphology and course of which predicts recurrence risk. These are currently categorized on radiological grounds and it is likely that they represent distinct pathological entities. Currently however there are no reliable means of differentiating those children with a monophasic disease course from those with recurrence or progressive arteriopathy. Circulating endothelial cells (CECs) are necrotic or highly activated endothelial cells that have become detached from the vessel wall, and have been recently shown to be a sensitive biomarker for detection of vascular injury and damage.

**Objective:** Test the hypothesis that it is possible to non-invasively track persistent endothelial injury (elevated CEC) in children with AIS recurrence compared to those with a single AIS event.

**Methods:** Single centre cross-sectional study of 46 children with AIS and cerebral arteriopathy matched with 20 paediatric controls. AIS recurrence was defined as new acute neurological deficit with radiological evidence of further cerebral infarction. Patients were considered in two groups based on AIS recurrence. CECs were identified using immunomagnetic bead extraction according to an international consensus protocol. Results were expressed as median and range.

**Results:** Forty six children with AIS aged 8.4 (0.9-17.4) years and 20 paediatric controls aged 9 (1.2-16) years were included. Ten children had AIS recurrence while 36 had a single AIS event. CECs were significantly raised in children with recurrent AIS, compared to those with no recurrence ( $p = 0.0001$ ); and controls,  $p= 0.0001$ . In two out of 8 children studied prospectively AIS recurrence was associated with a sustained increased in CECs. Six children had a monophasic disease course reflected on persistently low CEC counts. CEC as a biomarker of AIS recurrence was complimentary to current approaches based on radiological and clinical criteria.

**Conclusion:** Despite the wide spectrum of clinical and radiological presentation of childhood AIS indices of endothelial injury are different in patients with single and recurrent events. This novel observation has potential for furthering understanding of AIS pathophysiology and prognosis.

## **3.2 Introduction**

Childhood arterial ischaemic stroke (AIS) results in neurological morbidity in over 2/3rds of survivors and recurrence in up to 20% (Fullerton, Wu, Sidney, & Johnston 2007; Ganesan et al. 2006; Gordon, Ganesan, Towell, & Kirkham 2002). In the absence of acute treatments to limit brain injury, current approaches are focused on prevention of recurrence (Monagle et al 2004; Roach, 2008; Royal College of Physicians, 2004). The majority of affected children have arteriopathies of the cervical or intracranial circulation, which although radiologically similar, are likely to represent distinct pathological entities (Braun et al. 2009; Fullerton, Wu, Sidney, & Johnston 2007; Ganesan et al. 2003; Mackay, Wiznitzer, Benedict, Lee,

deVeber, & Ganesan 2011). The temporal association of VZV infection and arteriopathy as well as recent epidemiologic data from the International Paediatric Stroke Study (IPSS), suggest that common childhood infections may play a role in the genesis of this arteriopathy (Amlie-Lefond et al. 2009; Askalan et al. 2001; Sebire, Meyer, & Chabrier 1999). Other lines of evidence suggest that those affected could be genetically susceptible to generating an abnormal immunological response to such infections, resulting in vascular inflammation, cerebrovascular endothelial injury and a thrombogenic response (Amlie-Lefond & Fullerton 2010; Eleftheriou & Ganesan 2009; Fullerton et al. 2011; Kenet et al. 2010; Munot, Crow, & Ganesan 2011a). Although on radiological grounds both focal cerebral arteriopathy (FCA) and primary angiitis of the central nervous system (PACNS) are examples of arteriopathies that result in focal proximal occlusive disease of the intracranial arteries, the former is generally monophasic, while the latter has a recurrent course, possibly due to persistence of the aberrant inflammatory response described above, and potentially modifiable by steroids and immunosuppressants (Amlie-Lefond et al. 2009; Braun et al. 2009; Hutchinson, Elbers et al. 2010). However the distinction between these entities remains controversial (Braun et al. 2009; Eleftheriou & Ganesan 2009). Although previous studies have proposed radiological predictors of arteriopathy progression and AIS recurrence there is no clinically robust biomarker distinguishing between patients with a likely monophasic or a likely recurrent course (Braun et al. 2009; Cellucci et al. 2011). This inevitably leads to excessive or inadequate use of immunosuppression.

Endothelial cells adhere to a basement membrane and in health remain in this location with very few cells lost in the blood and cleared away through the reticuloendothelial system

(Sabatier et al. 2009). Pathological conditions associated with damage to the endothelium lead to endothelial cell detachment resulting in increased number of circulating endothelial cells (CEC) in the blood stream (Clarke, et al. 2010; Mutin et al. 1999; Smadja et al. 2009; Woywodt et al. 2002; Woywodt et al. 2003). The mechanisms involved in cell detachment are complex and are likely to involve several factors such as mechanical injury, atherosclerosis, alteration of cellular adhesion molecules, defective binding to anchoring matrix proteins, and cellular apoptosis with decreased survival of cytoskeletal proteins (Erdbruegger et al. 2006; Michel 2003; Sabatier et al. 2009). CECs are necrotic or highly activated endothelial cells that have become detached from the vessel wall in response to vascular injury (Clarke et al. 2010; Erdbruegger, Haubitz, & Woywodt 2006; Woywodt et al. 2003). CEC were initially detected in 1970 in smears of peripheral blood with techniques such as vital light microscopy (Hladovec and Rossmann 1973; Wright and Giacometti 1972). Cells were separated by Ficoll density centrifugation and identified based on their morphology and Giemsa staining (Hladovec & Rossmann 1973; Wright & Giacometti 1972). Immunohistochemistry of peripheral blood smears using antibodies to endothelial markers such as Von Willebrand Factor or cell adhesion molecules was employed years later (Jaffe 1987; Percivalle et al. 1993; Watanabe and Tokunaga 1990). However some smaller endothelial cell fragments were difficult to detect using this methodology and cell permeabilisation was necessary thus increasing cell loss (Blann et al. 2005). Overall these early studies were heavily reliant on morphology and were technically challenging. In addition, the poor specificity of these techniques and the low yield of cells complicated the research efforts in this area and highlighted the need for a reliable, endothelial-specific cell surface molecule that could be used as an appropriate tool (Blann et al. 2005).



Due to the low numbers of CEC in peripheral blood, an important step in their identification has since been the development of sensitive technologies for the detection of rare events based on immunolabelling with monoclonal antibodies to endothelial antigens (Blann et al. 2005). In 1991, two groups reported simultaneously monoclonal antibodies to new cell surface antigens on endothelial cells (HEC 19 and S-Endo 1) and used them to quantify blood-borne CECs (George et al. 1992; Sbarbati et al. 1991). Bardin et al. subsequently demonstrated that their antibody was recognizing the CD146 molecule and used it to demonstrate CECs in several conditions including rickettsial infection, sickle cell disease, thrombotic thrombocytopenia and acute coronary syndromes (Bardin et al. 1996a; Bardin et al. 1996b). This adhesion molecule is concentrated at the endothelial junction, where it plays a key role in the control of cell-cell cohesion, permeability, and signalization (Bardin et al. 2001). CD146 is more specific for endothelial cells (although it is found on some activated T-cells and melanoma cells) compared to other endothelial cell surface markers such as CD31 (also found on platelets, monocytes, neutrophils and T-cells), or CD105 (found on activated monocytes, macrophages and erythrocyte precursors) (Blann et al. 2005; Mutin et al. 1997).

In 1997, Solovey et al. used a monoclonal antibody (P1H12) against CD146 to detect CECs in sickle cell anaemia (Solovey et al. 1997). Immunomagnetic bead extraction has since become the consensus methodology for CEC identification and enumeration and is described in more detail in section 3.4.2 (Woywodt et al. 2003;Woywodt et al. 2006). The CECs are counted in whole blood using 4.5- $\mu$ m magnetic particles bound to an anti-CD146

monoclonal antibody (Woywodt et al. 2006). The use of an Fc-blocking agent (to prevent nonspecific leukocyte binding) and relatively endothelial-specific *Ulex europaeus* lectin 1 has improved the specificity of this technique (Woywodt et al. 2006). A CEC is defined as an event of appropriate size  $> 10 \mu\text{m}$  that binds (forms a rosette with) 5 or more beads (Woywodt et al. 2006).

Alternative methodologies for CEC counting include flow cytometry, which has been largely used to detect CEC in cancer patients amongst others (Bertolini et al. 2006; Fürstenberger et al. 2006; Mancuso et al. 2001; Rajagopalan et al. 2004; Sabatier et al. 2009). An advantage of flow cytometry is rapid multiparametric analysis, and the ability to detect sub-populations (Bertolini, Shaked, Mancuso, & Kerbel 2006; Fürstenberger et al. 2006; Mancuso et al. 2001; Rajagopalan et al. 2004; Sabatier et al. 2009). However, the methodology is not standardized with normal values using flow cytometry varying between publications and about 100–1000-fold higher compared to those from immunomagnetic bead extraction (Clarke et al. 2008; Shantsila and Lip 2008). These differences may be secondary to isolation procedure and inadequate standardization of flow cytometric conditions and are discussed in more detail in section 3.7.

Recently various groups have reported increased numbers of CECs in many diverse diseases including infectious (George et al. 1993) and cardiovascular diseases such as acute coronary syndromes (Boos et al. 2006; Mutin et al. 1999), diabetes (McClung et al. 2005), pulmonary hypertension (Smadja et al. 2009), chronic renal failure (Rodriguez-Ayala et al. 2006) and cancer (Mancuso et al. 2001). Low levels have been documented in healthy individuals of all

ages (Blann et al. 2005; Sbarbati et al. 1991). CECs are also increased in other pathological situations associated with vascular inflammation including lupus and systemic vasculitis (Clancy et al. 2001; Clarke et al. 2010; Woywodt et al. 2003). In various diseases, the longitudinal quantification of CECs has demonstrated that their levels vary according to the clinical condition/severity (Clancy et al. 2001; Clarke et al. 2010; Woywodt et al. 2003). In systemic vasculitis for instance Woywodt et al. have shown that patients with antineutrophil cytoplasmic antibody associated vasculitis (AAV) have increased numbers of CEC that decreased in response to 6 months of treatment to induce remission (Woywodt et al. 2003). Similarly Clarke et al. have shown in children with primary systemic vasculitis that CEC are a sensitive marker of disease activity and can be used to monitor response to therapy (Clarke et al. 2010). An important feature of CEC is that their levels are predominantly affected by disease activity and endothelial injury and not treatment per se (Clarke et al. 2010; Woywodt et al. 2003). Patients during vasculitis flares mounted a response by increasing their CECs despite receiving immunosuppressive treatment (Clarke et al. 2010; Woywodt et al. 2003). Interestingly, Nadar et al. recently studied adults who presented with an acute stroke and showed an increase in numbers of CECs in the acute phase correlating with other markers of endothelial dysfunction or damage such as soluble E-selectin and plasma vWF (Nadar et al. 2005). In addition, Woywodt et al. have also demonstrated high numbers of CEC during acute adult stroke (Woywodt et al. 2011). These clinical studies indicate that CEC levels are indicative of disease activity and generally correlate with functional or plasma markers of endothelial injury.

In summary, CECs have been used as markers of endothelial damage across an ever widening variety of diseases, including systemic vasculitis and adult stroke (Clarke et al. 2010; Nadar et al. 2005; Woywodt et al.2003; Woywodt et al. 2011). CECs could therefore be an attractive candidate biomarker to detect persistence of endothelial injury and how this relates to risk of recurrent childhood AIS.

### **3.3 Aims**

To explore whether children with recurrent AIS have evidence of increased endothelial injury as detected by raised CEC count compared with those with a single AIS event.

### **3.4 Methods**

#### ***3.4.1 Preparation of immunomagnetic beads***

Dynabeads (pan mouse IgG) were resuspended and then 350 µl of the suspension was added to a 5ml polypropylene tube. The tube used was then placed into the magnet (MPC-L; Dynal Biotech) for 2 minutes to allow the beads to attach to the side of the tube. The supernatant was discarded and the beads resuspended in 1ml phosphate buffered saline (PBS; Sigma) with 0.1% bovine serum albumin (BSA; Sigma) added. The tube was placed in the magnet again and the supernatant discarded as above. The beads were then resuspended in 950 µl of PBS (with 0.1% BSA) and 400 µl of s-endo-1 antibody (Biocytex) were added (final concentration of 25 µg/ml). The tube was sealed and the sample mixed on a roller mixer at 2

hours at 4<sup>0</sup> C. Beads were washed 3 times as before and resuspended in a final volume of 1 ml for further use.

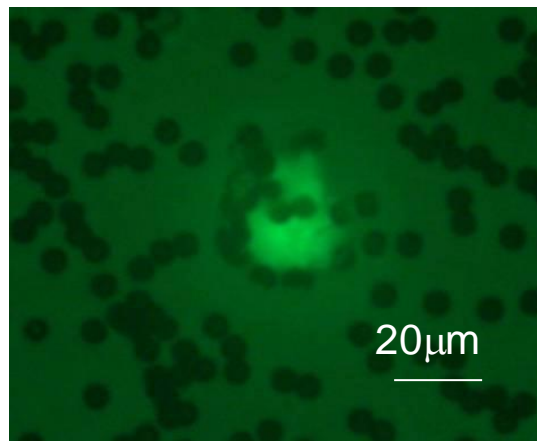
### **3.4.2 CEC extraction from peripheral blood**

CEC were detected as described previously based on an international consensus protocol (Woywodt et al. 2006). Briefly venous blood (1 ml) collected into tubes containing EDTA was mixed with buffer (1 ml of PBS containing 0.1% BSA and 0.6% sodium citrate) and 20µl of Fc receptor–blocking reagent (Miltenyi Biotec) and incubated for 5 minutes at room temperature. Fifty µl of a preparation of anti-CD146–coated immunomagnetic beads (clone S-endo-1; BioCytex and Dynal Biotech) was added, and the sample was incubated at 4°C for 30 minutes with rotation. Bead-bound cells were separated using a magnet (MPC-L; Dynal Biotech) and washed 3 times with buffer. Cells were then resuspended in 100 µl of buffer containing 10 µl of a 2-mg/ml preparation of FITC-labeled *Ulex europaeus* lectin (Sigma-Aldrich) and incubated for 1 hour at room temperature in the dark. Cells were then washed again three times and re-suspended in a final volume of 200 µl buffer.

### **3.4.3 Counting CEC**

CECs in the sample were counted using a Nageotte chamber on a fluorescence microscope. CECs were defined according to an international consensus protocol as Ulex bright cells that were >10 µm in size, with 5 magnetic beads attached, figure 3-1 (Woywodt et al. 2006).

Nageotte counting chamber is designed for counting of rare events and holds 50  $\mu\text{l}$  of sample over 40 lines. CEC were counted over the 40 lines and multiplied by 4 to get the total volume per ml of whole blood.



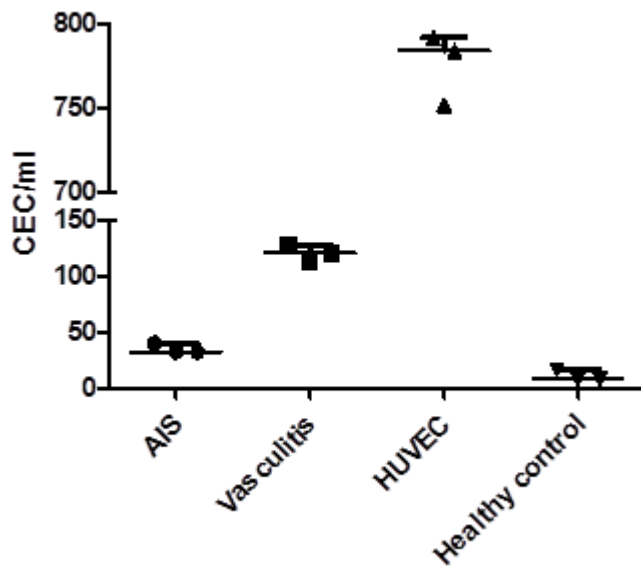
***Figure 3-1: Fluorescence microscopy image of a circulating endothelial cell (CEC) from a 4 year old child with cerebral arteriopathy and arterial ischaemic stroke.***

*CECs were separated from whole blood using immunomagnetic bead extraction, where cells bind to magnetic particles coated with anti-CD146 antibody and then are stained with FITC-conjugated Ulex europaeus lectin. CECs are defined as Ulex bright cells that were  $>10 \mu\text{m}$  in size, with a minimum of 5 magnetic beads attached.*

#### ***3.4.4 Reproducibility of the technique***

To establish the reproducibility of the immunomagnetic bead extraction technique, CEC from a child with AIS, one child with active systemic vasculitis (associated with increased endothelial injury) and a healthy child were prepared from 3 individual 1 ml blood samples.

As a positive control blood was spiked with HUVEC 1000 cells/ml and 3 samples of 1 ml each prepared. As depicted in figure 3-2 there was little variation in all samples measured with the greater variation in the spiked with HUVEC blood sample. For healthy control sample mean CEC count was 10 cells/ml SD 4 cell/ml; for AIS 34 cells/ml with SD 4 cells/ml; systemic vasculitis 120 cells/ml SD 8 cells/ml and for spiked blood with HUVEC 776 cells/ml with SD 21 cells/ml.

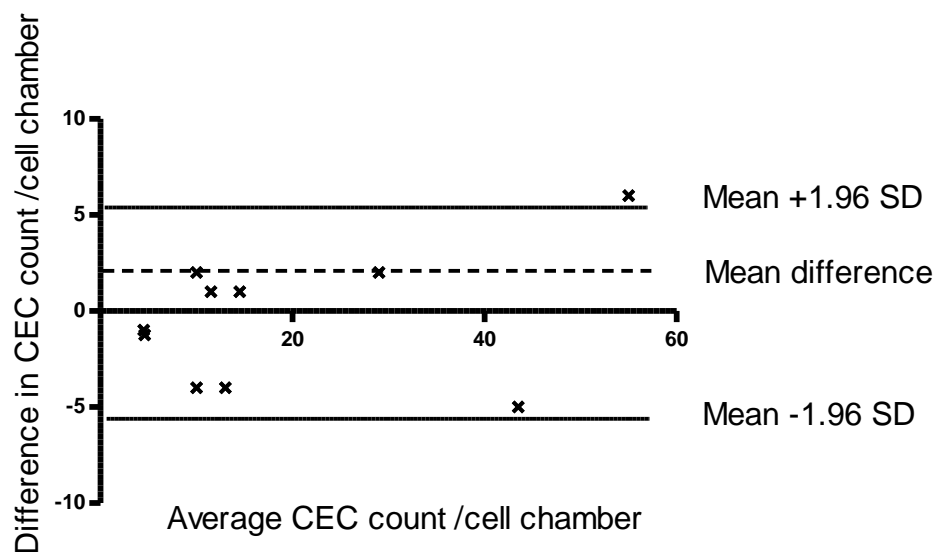


**Figure 3-2: Reproducibility of circulating endothelial cells with immunomagnetic bead extraction.**

Blood samples from a child with arterial ischaemic stroke (AIS), a child with systemic vasculitis and a healthy control sample were divided into 3 and circulating endothelial cells (CEC) counted. As a positive control a sample of healthy control blood was spiked with human umbilical vein endothelial cells (HUVEC) 1000 cells/ml. The mean CEC count in the healthy control sample was 10 cells/ml SD 4 cell/ml; for AIS 34 cells/ml with SD 4 cells/ml; systemic vasculitis 120 cells/ml SD 8 cells/ml and for spiked blood 776 cells/ml with SD 21 cells/ml.

### 3.4.5 Circulating endothelial cell enumeration-interobserver variability

Bland Altman plots of CEC counts (expressed as cells/cell chamber) for 10 consecutive patients with AIS and cerebral arteriopathy as counted by an experienced research fellow (Dr Ying Hong) and myself, both blinded to the classification of the patient are summarized in figure 3-3 below. Bland Altman analysis was performed to determine the inter-observer variability for CEC enumeration, showing a bias of -0.325 with SD 3.4 and 95% CI of agreement -7 to 6.



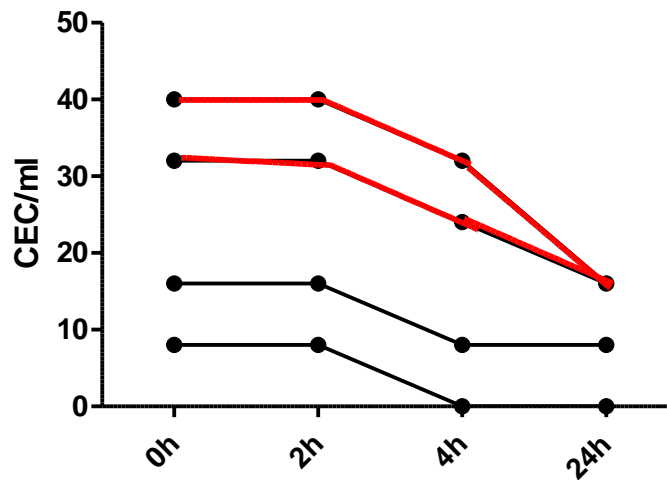
**Figure 3-3: Circulating endothelial cell (CEC) enumeration- interobserver variability.**

*Bland-Altman plot of CEC counts for 10 consecutive patients exploring the agreement between two blinded investigators for enumeration of CECs. Analysis shows a bias of -0.325 with SD 3.4 and 95% CI of agreement -7 to 6.*



### **3.4.6 Effect of time to preparation and storage on CEC enumeration**

Blood donated by an adult healthy volunteer and a sample from a child with AIS was divided into 1 ml aliquots and kept 4<sup>0</sup>C before processing and counting CEC at 2 hours, 4 hours and 24 hours. As previously described by Woywodt et al. CEC had declined by 24 hours in all samples (figure 3-4). Therefore all samples were processed immediately or within a 2 hour window and kept cold at 4<sup>0</sup> C.



**Figure 3-4: Effect of storage on circulating endothelial cell (CEC) counts.**

*Samples from an adult healthy volunteer and a child with arterial ischaemic stroke (marked in red) were kept at 4<sup>0</sup>C before processing at 2, 4 and 24 hours. By 24 hours CEC counts were decreased in all samples.*

## **3.5 Evaluation of circulating endothelial cells in children with arterial ischaemic stroke**

### ***3.5.1 Patient population and study design***

This was a cross-sectional study of children aged >28 days old with AIS and imaging evidence of cerebral/cervical arteriopathy presenting to Great Ormond Street Hospital (GOSH) from September 2007 to January 2012. Children/families were approached for recruitment to the study during their clinic follow up appointments or during inpatient stay at a median of 8 (6-24 months) following recent AIS. For definitions and inclusion/exclusion criteria see section 2.2. For healthy controls and disease controls see section 2.2.2. In addition to the cross sectional study, children with a new presentation of AIS and cervical/cerebral arteriopathy were evaluated prospectively over a 12 month period.

### ***3.5.2 Statistical analysis***

Numeric results were summarised as median and range. The Kruskal-Wallis test was used to examine overall differences in experimental laboratory markers between the study groups followed by the Mann-Whitney U test. Fisher's exact test was used to compare categorical data between groups. The Wilcoxon matched pairs signed ranks test was used to compare CEC counts at initial presentation and at latest follow up for those monitored prospectively. The independent association of CEC on the primary outcome of AIS recurrence was assessed using a multivariable logistic regression model, unadjusted and adjusted for age, gender, and time from stroke event to blood test sampling. Common confounding factors

such as age and sex but also time from stroke event to blood test sampling were entered into a univariate analysis initially and significant variables ( $p < 0.1$ ) were then entered into the multivariable model. Results were expressed as odds ratios (OR) with corresponding 95% confidence intervals (CI) and P-values. Sensitivity, specificity and likelihood ratios were then calculated to examine the potential diagnostic test characteristics of CECs for the identification of AIS recurrence. P-values of less than 0.05 (two sided) were regarded as significant. Statistical analysis was performed using SPSS version 17.

## **3.6 Results**

### ***3.6.1 Clinical, laboratory and radiological data***

A total 116 children with AIS were identified during the study period (figure 3-5). Of those 54 met the inclusion criteria; however eight patients/families refused participation. Of the 46 participants (age 8.8, range 0.9-17.4 years), 10 had AIS recurrence and 36 did not have AIS recurrence. The children who refused consent had an age of 6.2 (4.2-8.4 years old) and none had recurrent events. None of the children with a single AIS event had evidence of clinically silent infarction on repeat imaging at 6 and 12 months after index AIS. Healthy control children had an age of 9 (range 1.2-16) years old. The disease control group included 10 children median age 9 (3-16 years old) with cerebral AVM four of which had a history of intracerebral haemorrhage. The demographics of the study population, the routine clinical laboratory parameters, imaging features and arteriopathy classification are summarised in tables 3-1 and 3-2.

None of the children studied had clinical signs or symptoms of a systemic inflammatory disease. Evaluation took place at a median of 7 (6-13 months) following recent AIS for those children with AIS recurrence compared to 11 (6-24 months) for those with no recurrence, ( $p= 0.1000$ ). Recurrent AIS was confirmed at median of 7 (3-15 months) from index event. Considering the Sebire et al. classification there were 3 children with cervical arterial dissection (30%), one child with PACNS (10%), one child with moyamoya (10%), one child with PVA (10%) and 4 with an unclassified (40%) vasculopathy in the group of children with AIS recurrence. In the group of children with a single event there were 19 children with TCA/FCA (52%), 4 children with cervical arterial dissection (11%), 3 children with PVA (8%), 5 children with moyamoya (14.5%) and the remaining 5 (14.5%) had an unclassified vasculopathy. The CASCADE classification is provided for comparison in table 3-1.

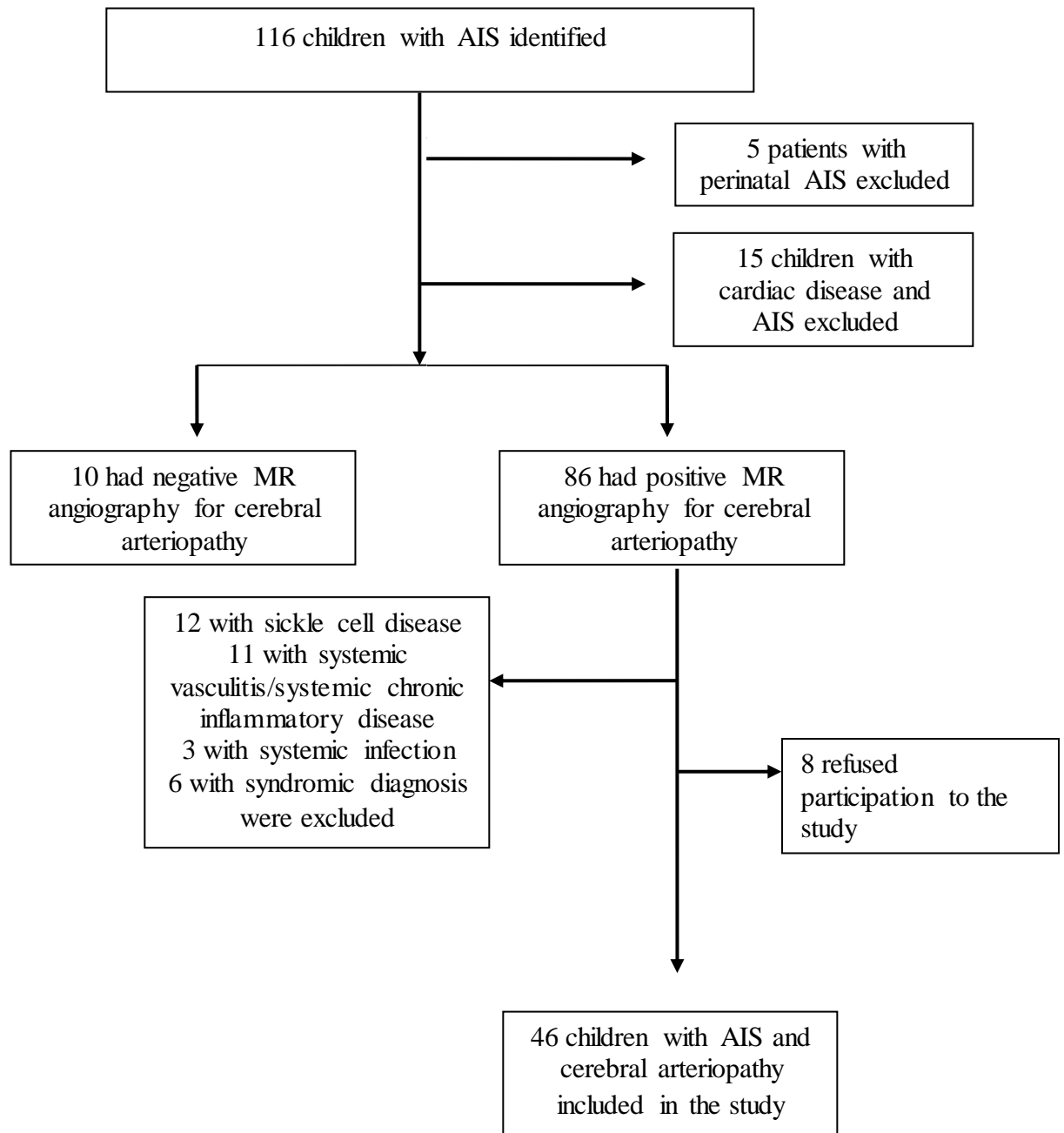
Of note, children with recurrent AIS more commonly had diffuse neurological deficits ( $p=0.0006$ ), headaches ( $p=0.0031$ ), multifocal and bilateral lesions ( $p=0.0002$ ), and lesions in both anterior and posterior circulation territories on MR imaging ( $p=0.0006$ ). They were also more likely to have been treated with steroids ( $p=0.0057$ ). Treatment with corticosteroids, cyclophosphamide and surgical revascularisation was initiated following AIS recurrence. In addition, there were no differences in ESR and CRP levels between the two groups ( $p=0.561$  and  $0.873$  respectively). With regards to thrombophilia screen one child in the group with recurrence was heterozygous for MTHFR mutations (10%) and one had initially low protein S (10%) that subsequently recovered to normal levels. In the group of children with no recurrence two patients had protein S deficiency (5%); one child was

MTHFR mutation homozygous (3%) with 3 being heterozygous (8%); lastly one child was heterozygous for factor V Leiden deficiency (3%).

Follow up MRI and MRA imaging data for the 10 children with AIS recurrence are summarised in table 3-3. All children had evidence of additional brain infarction. There was progression of previously identified arterial disease in 6 children (60%), involvement of previously unaffected vessels in 8 children (80%) and new arterial occlusion in the presence of other arterial wall changes in 2 patients (20%). Follow up MRI and MRA imaging data for the 36 children with no AIS recurrence are summarised in table 3-4.

Three out of the 10 children with AIS recurrence had cervical arteriopathy on initial MRA imaging. At the time of AIS recurrence all 3 children had evidence of progression of their cervical arteriopathy and in addition new evidence of intracranial arteriopathy. Five of the 36 children in the no recurrence group with cervical arteriopathy had no evidence of progression of this arteriopathy on follow up imaging and there was no new intra-cerebral circulation involvement identified.

In addition to the cross sectional study, 8 children, with age 6.5 (5-13 years) were studied prospectively at initial presentation with AIS and at latest follow up of 12 (9-18 months). Six had a monophasic disease course with no AIS recurrence following the initial event; while two children (treated with aspirin and warfarin but no immunosuppression) re-presented with AIS recurrence during follow-up (see table 3-5 and 3-6).



**Figure 3-5: Study profile.**

<b>Demographics</b>	<b>AIS recurrence n=10 (%)</b>	<b>AIS no recurrence n=36 (%)</b>	<b>P value</b>
Sex (M:F)	6:4	16:20	0.4839
Age (median, range) years	8.2 (0.9-15.4)	9.4 (2.7-17.4)	0.681
<b>Clinical features</b>			
Focal neurological deficit	10 (100%)	35 (97%)	1.0000
Diffuse neurological deficits	7 (70%)	4 (11%)	<b>0.0006</b>
Headache	5 (50%)	2 (5%)	<b>0.0031</b>
Seizures	1 (10%)	1 (3%)	0.3913
<b>Sebire et al. classification of cerebral arteriopathy (Sebire et al. 2004)</b>	N=3 Arterial dissection (30%) N=1 PACNS (10%) N=1 PVA (10%) N=1 Moyamoya (10%) N= 4 Unclassified (40%)	N=19 TCA/FCA (52%) N=4 Arterial dissection (11%) N=3 PVA (8 %) N=5 Moyamoya (14%) N=5 Un-classified (14%)	N/a
<b>CASCADE primary classification (Bernard et al. 2012)</b>	N=3 Aortic/cervical arteriopathy (30%) N=1 Small vessel arteriopathy of childhood (10%) N=1 Unilateral FCA (10%) N=4 Bilateral arteriopathy with collaterals (40%) N=1 Bilateral arteriopathy without collaterals (10%)	N=22 Unilateral FCA (61%) N=4 Aortic/cervical arteriopathy (11%). N=5 Bilateral arteriopathy with collaterals (14%) N=3 Bilateral arteriopathy without collaterals (8%) N=2 Other (5%)	N/a
<b>ESR mm/h (median, range) (normal range 0-10 mm/h)</b>	5.4 (1-12) mm/h	6.6 (1-28) mm/h	0.561
<b>CRP mg/L (median, range) (normal range &lt;10 mg/L)</b>	5 (3-16) mg/L	5 (3-6) mg /L	0.873
<b>Cerebrospinal fluid analysis</b>	Normal (6/6 studies)	Normal (n=5/5 studies)	1
<b>Thrombophilia screen</b>	Any abnormality n=2 (20%)  MTHFR heterozygous n=1 (10%) Low protein S n=1 (10%)	Any abnormality n=7 (19%) Protein S deficiency n=2 (5%) MTHFR homozygous n=1 (3%) Heterozygous n=3 (8%)	1

		Factor V Leiden heterozygous n=1 (3%)	
<b>Treatment</b>			
Aspirin	10 (100%)	36 (100%)	1
Anticoagulation (heparin/warfarin)	3 (30%)	5 (14%)	<b>0.0149</b>
Corticosteroids	4 (40%)	1 (3%)	<b>0.0057</b>
Cyclophosphamide	2 (20%)	1 (3%)	0.1147
Surgical revascularization	2 (20%)	4 (11%)	0.5975

**Table 3-1: Study population characteristics.**

*Focal neurological deficits included hemiparesis, facial weakness and hemisensory loss. Diffuse neurological deficits included neurocognitive dysfunction, personality changes and concentration difficulties. M=male; F=Female; TCA=Transient cerebral arteriopathy; FCA=Focal cerebral arteriopathy; PACNS=primary angitis of the central nervous system; ESR=erythrocyte sedimentation rate; CRP=C-reactive protein; MTHFR= Methylene-tetrahydrofolate reductase; PVA=Post varicella arteriopathy; N/a=not applicable. P values were calculated using Fisher's exact test for categorical data and Mann Whitney U for continuous variables. P-values of less than 0.05 (two sided) were regarded as significant.*



<b>Imaging modality</b>	<b>AIS recurrence n=10 (%)</b>	<b>AIS no recurrence n=36 (%)</b>	<b>P value</b>
<b>MRI</b>			
Multifocal and bilateral lesions	9 (90%)	8 (22%)	<b>0.0002</b>
<b>MRA</b>			
Multifocal and bilateral lesions	9 (90%)	8 (22%)	<b>0.0002</b>
Proximal lesions	10 (100%)	36 (100%)	1
Distal lesions	8 (80%)	3 (8%)	<b>0.0001</b>
Anterior circulation only	2 (20%)	30 (83%)	<b>0.004</b>
Posterior circulation only	2 (20%)	6 (16%)	1
Anterior and posterior circulation	7 (70%)	4 (11%)	<b>0.0006</b>
Collaterals	4 (40%)	3 (8%)	<b>0.0311</b>
<b>Catheter angiography (total patients)</b>	10	14	
Multifocal and bilateral lesions	9 (90%)	8 (57%)	0.1718
Anterior and posterior circulation	7 (70%)	6 (43%)	0.2397

***Table 3-2: Imaging findings for 46 children with arterial ischaemic stroke (AIS).***

*MRI= magnetic resonance imaging; MRA=magnetic resonance angiography. P values were calculated using Fisher's exact test for categorical data and Mann Whitney U for continuous variables. P-values of less than 0.05 (two sided) were regarded as significant.*

<b>Magnetic resonance imaging (MRI)</b>	<b>Patients N=10 (100%)</b>
Additional brain infarction	10 (100%)
<b>Magnetic resonance angiography (MRA)</b>	
Progression of previously identified arterial disease	6 (60%)
Involvement of previously unaffected vessels	8 (80%)
New arterial occlusion in the presence of other arterial wall changes	2 (20%)

*Table 3-3: Magnetic resonance imaging and angiography findings in 10 children with arterial ischaemic stroke (AIS) recurrence.*

<b>Magnetic resonance imaging (MRI)</b>	<b>Patients N=36 (100%)</b>
Additional brain infarction	0 (0%)
<b>Magnetic resonance angiography (MRA)</b>	
Progression of previously identified arterial disease	4 (11%)
Involvement of previously unaffected vessels	0 (0%)
New arterial occlusion in the presence of other arterial wall changes	0 (0%)

*Table 3-4: Magnetic resonance imaging and angiography findings in 36 children with no arterial ischaemic stroke (AIS) recurrence.*

<b>Demographics</b>	<b>Recurrence n=2 (%)</b>	<b>No recurrence n=6 (%)</b>	<b>P value</b>
Sex (M:F)	2:0	4:2	1
Age (median, range) years	8 (6-12)	7 (6.5-13)	0.581
<b>Clinical features</b>			
Focal neurological deficit (hemiparesis, facial weakness, hemisensory loss)	2 (100%)	6 (100%)	1
Diffuse neurological deficits (neurocognitive dysfunction, personality changes, concentration difficulties)	2 (100%)	1 (50%)	0.1
Headache	2 (100%)	0 (0%)	<b>0.03</b>
Seizures	1 (50%)	0 (0%)	0.3913
<b>Sebire et al. classification of cerebral arteriopathy (Sebire et al. 2004)</b>	N=2 unclassified (40%)	N=2 TCA/FCA (33%) N=2 arterial dissection (33%) N=1 PVA (16%) N=1 un-classified (16%)	N/a
<b>CASCADE primary classification (Bernard et al. 2012)</b>	N=1 Bilateral arteriopathy with collaterals (50%) N=1 Bilateral arteriopathy without collaterals (50%)	N=3 Unilateral FCA (50%) N=2 Aortic/cervical arteriopathy (33%) N=1 Bilateral arteriopathy without collaterals (16%)	N/a

<b>ESR mm/h (median, range)</b> <b>(normal range 0-10 mm/h)</b>	15 (1-20) mm/h	10 (1-25) mm/h	0.478
<b>CRP mg/L (median, range)</b> <b>(normal range &lt;10 mg/L)</b>	10 (3-20) mg/L	5 (3-10) mg /L	0.561
<b>Treatment</b>			
Aspirin	2 (100%)	6 (100%)	1
Anticoagulation (heparin/warfarin)	2 (100%)	1 (16%)	0.1
Corticosteroids	0 (0%)	0 (0%)	1
Cyclophosphamide	0 (0%)	0 (0%)	1
Surgical revascularization	0 (0%)	0 (0%)	1

**Table 3-5: Characteristics of the population studied prospectively.**

*Focal neurological deficits included hemiparesis, facial weakness and hemisensory loss. Diffuse neurological deficits included neurocognitive dysfunction, personality changes and concentration difficulties. M=male; F=Female; TCA=Transient cerebral arteriopathy; FCA=Focal cerebral arteriopathy; PACNS=primary angiitis of the central nervous system; ESR=erythrocyte sedimentation rate; CRP=C-reactive protein; MTHFR= Methylene-tetrahydrofolate reductase; PVA=Post varicella arteriopathy; N/a=not applicable. P values were calculated using Fisher's exact test for categorical data and Mann Whitney U for continuous variables. P-values of less than 0.05 (two sided) were regarded as significant.*

<b>MRI</b>			
Multifocal and bilateral lesions	2 (100%)	1 (16%)	0.1
<b>MRA</b>			
Multifocal and bilateral lesions	2 (100%)	1 (16%)	0.1
Proximal lesions	2 (100%)	6 (100%)	1
Distal lesions	2 (100%)	0 (0%)	<b>0.03</b>
Anterior circulation only	0 (0%)	4 (66%)	0.42
Posterior circulation only	0 (0%)	2 (33%)	1
Anterior and posterior circulation	2 (100%)	0 (0%)	<b>0.03</b>
Collaterals	1 (50%)	0 (0%)	0.25
<b>Catheter angiography(total patients)</b>	2 (100%)	1 (16%)	0.1
Multifocal and bilateral lesions	2 (100%)	0 (0%)	0.33
Anterior and posterior circulation	2 (100%)	0 (0%)	0.33
Collaterals	1 (50%)	0 (0%)	1

***Table 3-6: Imaging findings for population studied prospectively.***

*MRI= magnetic resonance imaging; MRA=magnetic resonance angiography. P values were calculated using Fisher's exact test for categorical data and Mann Whitney U for continuous variables. P-values of less than 0.05 (two sided) were regarded as significant*

### ***3.6.2 Circulating endothelial cells track endothelial injury in childhood***

#### ***AIS recurrence***

Levels of peripheral blood CEC were significantly higher in 46 children with AIS and cerebral/cervical arteriopathy (median count 40/ml, range 0-736) compared to 20 healthy controls (median count 24/ml, range 0–60) in  $p=0.03$  (figure 3-6A). The CEC count in the

10 children with AIS recurrence was significantly higher at 120/ml (64-732) compared to 32/ml (0-64) in the 36 children with no recurrence ( $p = 0.0001$ ); 24/ml (0-60) in 20 child controls ( $p=0.0001$ ); and in 10 children with cerebral AVM ( $p=0.0002$ ) (figure 3-6B). There were no significant differences between CEC in children with AIS and a single event compared to controls ( $p= 0.188$ ) and children with cerebral AVM ( $p=0.117$ ).

The study population was then considered into 2 groups based on arteriopathy progression (progression of previously identified arterial disease; new arterial disease defined as involvement of previously unaffected vessels; new arterial occlusion in the presence of other arterial wall changes). Figure 3-7 shows the differences between CEC counts in children with progressive arteriopathy ( $n=14$ ) median 92/ml (40-736) compared to those children with no progressive arteriopathy ( $n=32$ ) median 24/ml (0-60),  $p=0.0001$  and healthy controls 24/ml (0-60),  $p=0.0001$ . Patients were also considered in two groups based on the possible inflammatory nature of the arteriopathy: the group of “presumed” inflammatory arteriopathies (TCA/PVA/PACNS) and the “presumed” non-inflammatory arteriopathies (moyamoya, dissection, unclassified). Twenty four children were included in the first group with the remaining 22 in the second group. Median CEC count in the presumed inflammatory arteriopathy group was 36 (0-736) cells/ml compared to a median of 64 (0-392) cells/ml in the presumed non inflammatory arteriopathy group ( $p=0.320$ ; figure 3-8).

To ascertain the statistical power of CEC as a test to detect differences between study groups given the small number of healthy controls recruited a retrospective power calculation was conducted. Based on a sample size of 20 healthy control children, a mean CEC of 30

cells/ml for the control group and mean CEC of 70 cells/ml for the group of children with AIS, SD 18 cells/ml and an observed value of  $p=0.03$  revealed a power of 90% for this study.

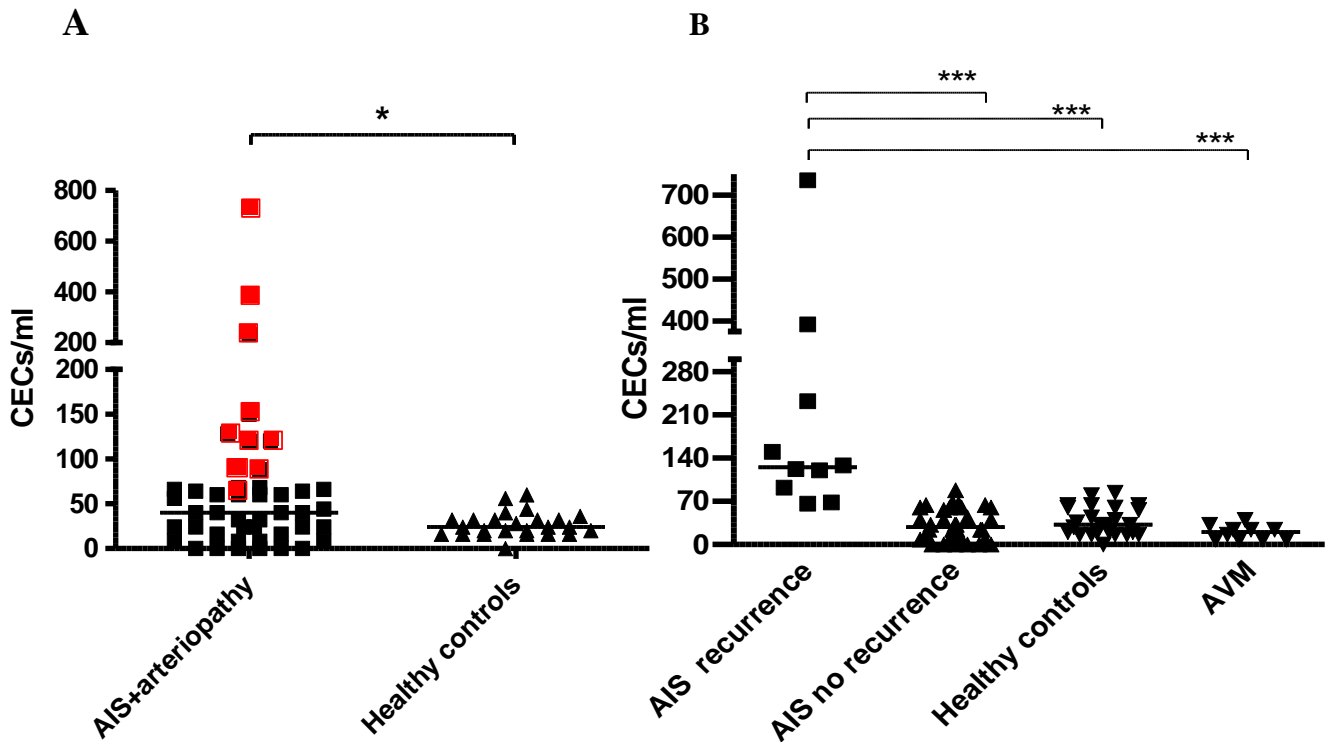
The phenotype of the 2 children with the highest CEC counts (736 cells/ml and 392 cells/ml) is described below:

**Case 1.** A healthy 14-year-old male with no history of trauma or infection presented with acute left hemiparesis secondary to right basal ganglia infarct and evidence of right MCA focal arteriopathy. Additional investigations were normal (echocardiogram, CRP, ESR, antinuclear antibodies, antineutrophil cytoplasmic antibodies, anticardiolipin antibody, lupus anticoagulant, complement, immunoglobulins, varicella zoster virus serology, and thrombophilia screening) and CSF revealed no pleocytosis or presence of oligoclonal bands. He was then started on aspirin therapy. Nine months later he represented with a right sided hemiparesis. Repeat MRI brain revealed left posterior lentiform nucleus, left thalamus, right caudate and periventricular region white matter lesions. Vascular imaging including MRA and conventional angiography revealed arteriopathy with irregular areas of stenosis and dilatation of the left common and lateral segment of the left internal carotid, both of the anterior cerebral and middle cerebral arteries proximally and distally and posterior circulation involvement without dissection or collaterals. On suspicion of cerebral vasculitis and severe progression of arteriopathy with multiple recurrent strokes, he was treated with IV methylprednisolone (30 mg/kg, 3 days) followed by prednisolone (1 mg/kg initially, 6 month wean) and monthly cyclophosphamide (500 mg-750 mg/m<sup>2</sup> per dose; total of 6

doses). At 18 months, repeat MRI brain showed maturation of the previously noted infarcts and no new lesion.

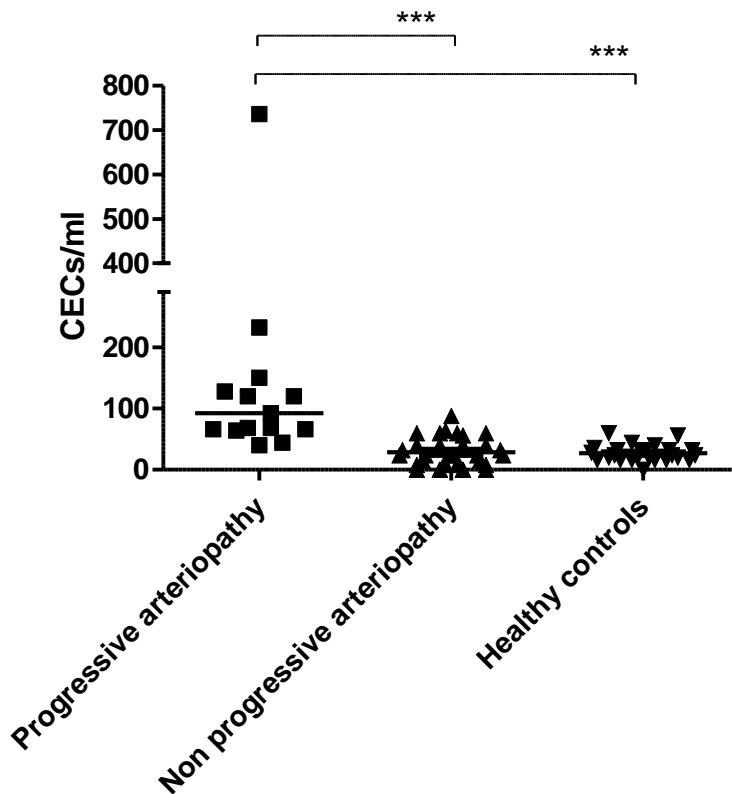
**Case 2.** A 3 year old boy with a history of mild fine and gross motor skill developmental delay presented with acute right hemiparesis secondary to left MCA infarction. Vascular imaging including MRA and conventional angiography revealed stenosis of the left internal carotid artery and left proximal MCA. Additional investigations were normal (echocardiogram, CRP, ESR, antinuclear antibodies, antineutrophil cytoplasmic antibodies, anticardiolipin antibody, lupus anticoagulant, complement, immunoglobulins, varicella zoster virus serology, and thrombophilia screening). His arteriopathy was best classified as FCA and he was started on aspirin. Four months later he re-presented with a new episode of right sided hemiparesis and seizures. Repeat imaging revealed progression of the arteriopathy in the left ICA and MCA both in length and severity and new involvement of the A1 segment; normal posterior circulation no dissection or collaterals identified. Anticoagulation with warfarin was added to his therapy. He subsequently had 4 further episodes of acute neurological presentation associated with AIS recurrence and further arteriopathy progression (new stenosis of the right ICA, MCA and vertebral arteries irregularity) suggesting a multifocal vasculopathy. Two years following his initial presentation he successfully underwent an EC-IC by pass and remains currently stable.





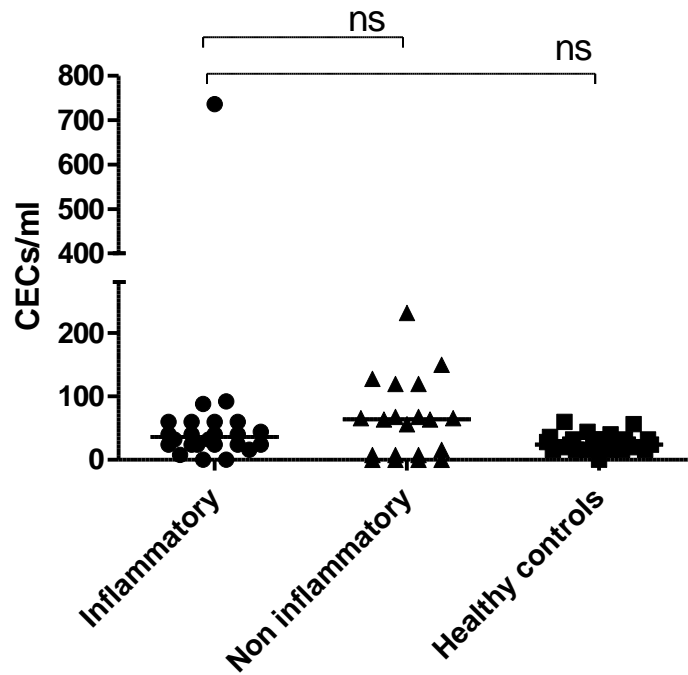
**Figure 3-6: Circulating endothelial cells (CECs) non-invasively track ongoing endothelial injury in children with recurrent arterial ischaemic stroke (AIS).**

(A) CEC were significantly higher in children with AIS and cerebral/cervical arteriopathy compared to healthy controls,  $p=0.03$ . CEC counts for children with recurrent AIS are marked in red. (B) CEC count in children with recurrent AIS was significantly higher compared to children with no recurrence,  $p = 0.0001$ ; children with cerebral arterio-venous malformation (AVM),  $p=0.0002$ ; and child controls,  $p=0.0001$ . The Kruskal-Wallis test followed by the Mann-Whitney U test was used to examine differences between the study groups. \* $P<0.05$ , \*\* $P< 0.005$ , \*\*\* $P< 0.0005$  (two sided).



**Figure 3-7: Circulating endothelial cells (CECs) in children with progressive arteriopathy and arterial ischaemic stroke (AIS).**

CEC were significantly higher in children with AIS and cerebral arteriopathy progression compared to those with no progressive arteriopathy ( $p=0.0001$ ) and healthy controls ( $p=0.0001$ ). The Kruskal-Wallis test followed by the Mann-Whitney U test was used to examine differences between the study groups. \* $P<0.05$ , \*\* $P<0.005$ , \*\*\* $P<0.0005$  (two sided).

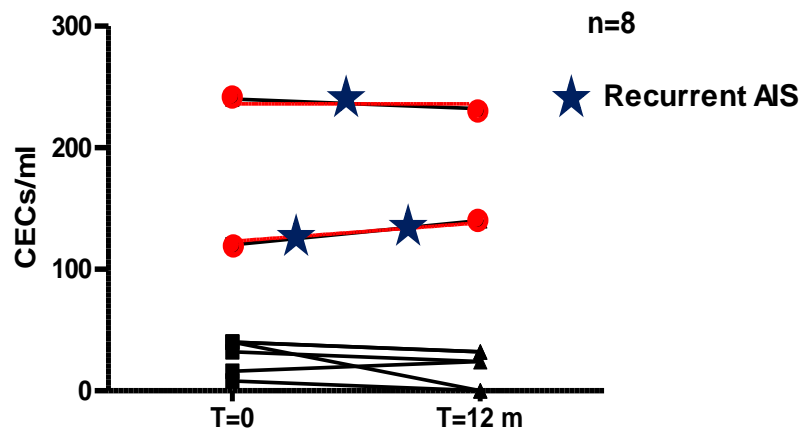


**Figure 3-8: Circulating endothelial cells (CECs) in children with presumed inflammatory cerebral arteriopathy and arterial ischaemic stroke (AIS).**

*Focal cerebral arteriopathy, post varicella arteriopathy and primary central nervous system vasculitis were considered as inflammatory arteriopathies. Moyamoya arteriopathy, arterial dissection and other vasculopathies were considered non-inflammatory. There were no significant differences in CEC between children with AIS and presumed inflammatory cerebral arteriopathy (n=24) and those with presumed non-inflammatory arteriopathy (n=22; p=0.375) and healthy controls, p=0.05. The Kruskal-Wallis test followed by the Mann-Whitney U test was used to examine differences between the study groups.*

### ***3.6.3 Longitudinal changes in CEC in children with arterial ischaemic stroke recurrence and cerebral arteriopathy***

For 8 children CEC were prospectively assessed at initial presentation with AIS and at latest follow up of median 12 months (9-18 months). Six of these children had a monophasic disease course with no recurrence following the initial event and were treated with aspirin alone. CEC at presentation for these 6 children were 36 (8-40) cells/ml and at follow up 24 (0-32) cells/ml ( $p=0.1198$ ). For 2 children there was clinical AIS recurrence associated with cerebral arteriopathy progression. One child had recurrence 6 months after the initial AIS while the other child had 2 recurrences at 2 and 8 months following initial presentation. The CEC counts for these children with AIS recurrence was raised at 180 (120-240) cells/ml at presentation and remained high at 186 (140-232) cells/ml at 12 months follow up (figure 3-9). These two children were treated with aspirin and additional anticoagulation (heparin and warfarin) following recurrence.



**Figure 3-9: Prospective changes in circulating endothelial cell (CEC) count in 8 children with arteriopathy and arterial ischaemic stroke (AIS).**

CECs at presentation for 6 children with a monophasic disease course were 36 (8-40) cells/ml and at follow up 24 (0-32) cells/ml. For 2 children there was clinical AIS recurrence associated with cerebral arteriopathy progression (shown in red). The CEC counts for these children was raised at 180 (120-240) cells/ml at presentation and remained high at 184(136-232) cells/ml at 12 months follow up.

### **3.6.4 Logistic regression analysis of CEC for AIS recurrence**

Logistic regression analysis was used to examine the relationship of CECs to AIS recurrence. Table 3-7 gives the unadjusted odds ratios, and odds ratios adjusted for common confounding factors such as age and gender. As the time from recent AIS event to blood sampling was shorter in the group of children with recurrent AIS (median 7, range 6-13 months) compared to those with a single event (median 11, range 6-24 months) and could affect the relationship of CEC to the primary outcome of AIS recurrence, this parameter was also examined in the regression analysis. CECs were significantly associated with AIS recurrence in the unadjusted analysis (OR 2.238; 95% CI 2.060-2.445, p= 0.005) and this association remained significant in the adjusted analysis (OR 2.021; 95% CI 2.004-2.038, p= 0.016).

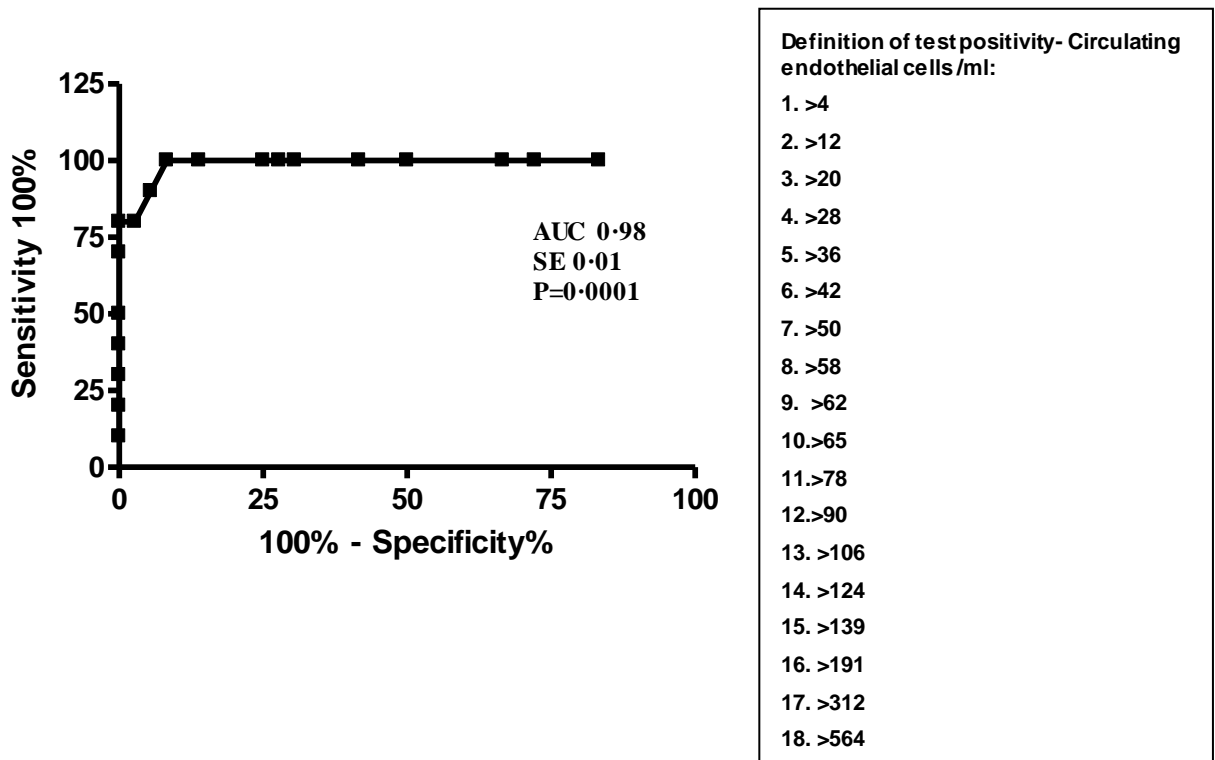
<b>Biomarker</b>	<b>Unadjusted</b>			<b>Adjusted (age/gender/time from AIS to evaluation)</b>		
	<b>OR</b>	<b>95%CI</b>	<b>P</b>	<b>OR</b>	<b>95%CI</b>	<b>P</b>
Circulating endothelial cells (CECs)	2.438	2.060-2.645	0.005	2.021	2.004-2.038	0.016

**Table 3-7: Unadjusted and adjusted odds ratios by multivariable logistic regression analysis for arterial ischaemic stroke recurrence (AIS).**

*Adjusted odd ratios for age/gender/time from AIS to evaluation; OR=odds ratio; CI=confidence intervals. P<0.05 was considered significant.*

### ***3.6.5 Test characteristics of CEC for identification of children at risk of AIS recurrence***

On the basis of this multivariable analysis demonstrating that high CECs strongly associated with recurrent AIS, the test characteristics of CECs for identification of AIS recurrence by plotting ROC curves were then examined. The ROC curve for CECs at varying definitions of positivity is shown in figure 3-10. ROC analysis for CECs as a diagnostic test for identification of recurrence was significant with an AUC 0.98, SE 0.01 and 95% CI of 0.95-1.000,  $p=0.0001$ . The full test characteristics of CECs for identification of AIS recurrence are summarized in table 3-8. We then considered the radiological features (arterial occlusion and/or arteriopathy progression) predicting AIS recurrence described by Braun and colleagues (Braun et al. 2009) and examined whether the additional assessment of CECs increased the sensitivity and/or specificity of identifying children with recurrence (table 3-8). A pre-test probability for AIS recurrence was based on a childhood AIS recurrence rate of 19% as reported in previous studies (Fullerton, Wu, Sidney, & Johnston 2007). Considering the Braun et al. radiological criteria alone, the post-test probability for correctly identifying patients with recurrence within this cohort was 67% (95% CI 44-79%). The combination of these radiological criteria and a CEC count cut off of  $> 64$  cells/ml (above the upper limit of healthy control CEC counts) resulted in a post-test probability of 86% (95% CI 55-94%). Thus, inclusion of the CEC count enhanced the predictive potential of the existing radiological criteria for identification of those at risk of AIS recurrence.



*Figure 3-10: Receiver operator characteristic (ROC) curve for circulating endothelial cells (CEC) as a diagnostic test for arterial ischaemic stroke (AIS) recurrence.*

*Definitions of test positivity were considered at varying levels of CEC counts as shown here.*

*ROC analysis was significant with area under the curve (AUC) of 0.98, SE 0.01 and 95% CI of 0.95-1.000, p=0.0001. P-values of less than 0.05 (two sided) were regarded as significant.*



<b>CEC level Cutoff</b>	<b>Sensitivity%</b>	<b>95% CI</b>	<b>Specificity%</b>	<b>95% CI</b>	<b>LR+</b>	<b>LR-</b>	<b>Post-test probability %</b>	<b>95%CI</b>
> 4	100	72-100%	17	7-34%	1.2	0	22%	18-25%
> 12	100	72-100%	29	15-46%	1.4	0	25%	20-26%
> 20	100	72-100%	34	19- 52%	1.5	0	26%	21-31%
> 28	100	72-100%	50	34-69%	2.06	0	32%	24-39%
> 36	100	72-100%	58	42 -76%	2.4	0	36%	27-42%
> 42	100	72-100%	69	54-85%	3.22	0	43%	31-53%
> 50	100	72-100%	74	57-88%	3.60	0	47%	33-59%
> 58	100	72-100%	75	60-90%	4.00	0	48%	34-63%
> 62	100	72-100%	86	73-97%	7.20	0	63%	42-81%
> 65	100	72-100%	91	81-99%	12	0	72%	47-85%
> 67	81	48- 98%	94	81- 99%	16.20	0.2	75%	48-92%
> 78	80	39- 94%	97	85- 99%	29	0.21	86%	52-97%
> 90	80	39 -94%	100	90- 100%	Infinity	0.2	100%	49-100%
> 106	70	31-89%	100	90 -100%	Infinity	0.3	100%	46-100%
> 124	50	17-77%	100	90-100%	Infinity	0.5	100%	35-100%
> 139	40	11-69%	100	90- 100%	Infinity	0.6	100%	32-100%
> 191	30	6- 61%	100	90-100%	Infinity	0.7	100%	26-100%
> 312	20	2- 52%	100	90- 100%	Infinity	0.8	100%	19-100%
> 564	10	0.2-41%	100	90 -100%	Infinity	0.9	100%	10-100%

***Table 3-8: Test characteristics of circulating endothelial cells (CEC) for identification of arterial ischaemic stroke (AIS) recurrence.***

*LR+ = Likelihood ratio for a positive test result LR-=Likelihood ratio for a negative test result CI confidence intervals CEC=Circulating endothelial cells. Pre-test probability was based on a childhood arterial ischaemic stroke (AIS) recurrence rate of 19% as per previous publications( Fullerton et al. 2007).*

<b>Profile/test</b>	<b>Sensitivity (95% CI)</b>	<b>Specificity (95% CI)</b>	<b>LR+ (95% CI)</b>	<b>LR- (95% CI)</b>	<b>Post-test probability (95% CI)</b>
Radiological criteria† (Braun, et al. 2009)	100 (68- 100)%	88 (65-90)%	8.33 (3.34-16)	0 (0-0.79)	67 (44-79)%
CECs alone	100 (68- 100)%	90 (75-96)%	10 (3.65-21)	0 (0-0.77)	70 (46-83)%
CECs and radiological criteria† (Braun, et al. 2009)	100 (68- 100)%	96 (81 -99)%	25 (45.05-70)	0 (0-0.72)	86 (55-94)%

***Table 3-9: Circulating endothelial cell counts (CECs) and radiological features identify high risk profile of children at risk of arterial ischaemic stroke (AIS) recurrence.***

*Circulating endothelial cell (CEC) testing was considered positive at a cut off of > 64 cells/ml of whole blood (above the upper limit of CEC counts for healthy controls studied here). Pre-test probability was based on a childhood arterial ischaemic stroke (AIS) recurrence rate of 19% as per previous publications (Fullerton, Wu, Sidney, & Johnston 2007). LR+=Likelihood ratio for a positive test; LR-=likelihood ratio for a negative test; CI=Confidence interval. † Radiological criteria: arterial occlusion and/or arteriopathy progression (Braun et al. 2009).*

### **3.7 Discussion**

In this cross sectional study of a group of children with AIS and cerebral arteriopathy, CEC counts were identified as a biomarker that tracks endothelial injury and differs significantly between children with recurrent AIS and those with a monophasic disease course. CECs are a non-invasive surrogate marker of vascular injury that may be useful to stratify stroke recurrence risk in children with AIS and cerebral arteriopathy.

AIS in children is increasingly recognised as an important cause of lifelong morbidity, with personal, social and economic consequences (Ganesan et al. 2000; Goldenberg et al. 2009; Gordon, Ganesan, Towell, & Kirkham 2002; MacGregor, Curtis, & Mayank 2000). Since the advent of noninvasive cerebral arterial imaging, in particular MRA, it has become evident that cerebral arterial abnormalities are found in between 50 and 80% of these children (Ganesan et al. 2003; Mackay et al. 2011). The morphology and course of these cerebral/cervical arteriopathies are the most important determinants of recurrence thus far identified in childhood AIS (Fullerton, Wu, Sidney, & Johnston 2007; Ganesan et al. 2006). Current classifications are radiological, although it is well recognized that MRA and catheter angiography have limitations in identifying and differentiating between distinct pathological entities (Aviv et al. 2006; Bernard et al. 2012; Eleftheriou et al. 2010; Sebire, Fullerton, Riou, & deVeber 2004). In this study patients were classified based solely on AIS recurrence (corroborated both clinically and radiologically) as this is a robust, verifiable and clinically important event. Furthermore, progression of cerebral arteriopathy and AIS recurrence is most likely multifactorial but the degree and extent of involvement of inflammation,

endothelial injury and hyper-coagulability predisposing to and/or exacerbating acute ischaemic brain injury and accelerating the progression of cerebral arterial disease is largely undefined. As a result, distinguishing between patients likely to have a static or progressive course on radiological and clinical grounds at initial presentation remains challenging leading to variation in current management approaches. In addition, secondary preventative treatments are empiric, rather than specifically targeted to underlying disease (Monagle et al 2004; Roach, 2008; Royal College of Physicians, 2004).

CECs are matured differentiated cells that are shed from the vessel wall as a result of pathophysiologic conditions that affect the endothelium (Blann et al. 2005; Woywodt et al. 2003). CEC are present at very low levels in healthy subjects, representing physiological endothelial turnover, whereas elevated levels have been reported in several pathologic situations including cardiovascular diseases, immune-mediated disorders, infectious diseases, cancer and systemic vasculitis (Blann et al. 2005; Sabatier et al. 2009). This study now suggests that elevated CECs, and therefore persistence of endothelial injury vary between children with single and recurrent AIS. CECs could therefore be an attractive candidate biomarker to detect those at risk of recurrent AIS, a finding that is consistent with the strong correlation between CECs and disease activity in patients with systemic vasculitis (Clarke et al. 2010; Woywodt et al. 2003). Of note the CEC count, even when corrected for time of sampling from AIS event as this could be an important confounding factor, remained a significant predictor of AIS recurrence in a multivariable logistic regression model. Furthermore, in keeping with previous studies in systemic vasculitis, the few children studied (n=4) who were receiving immunosuppression at the time of AIS recurrence were

able to mount a high CEC response, indicating that CECs are a sensitive marker of endothelial injury and disease activity irrespective of therapy. Treatment would be in general expected to modify these biomarkers but when this is failing and endothelial injury occurs despite treatment, this can still be detected using CEC counts.

The findings reported here have several implications. First, they suggest that despite the wide spectrum of presentations of this heterogeneous condition and the lack of conventional circulating acute phase reactants, indices of endothelial injury can for the first time clearly separate disease groups with regards to AIS recurrence. In particular in this cross-sectional study CEC count was complementary to current radiological criteria in identifying patients with AIS recurrence. The preliminary prospective data presented herein additionally support the prognostic role of CEC. These observations if validated in larger prospective cohorts may enable a stratified approach, targeting therapy at those children with high risk of recurrence. Furthermore, our ability to study the endothelium non-invasively by studying CECs provides us with invaluable insights into the pathophysiology of childhood AIS. This could potentially at the individual patient level enable therapeutic options to integrate the predominant pathogenic mechanism. Those children for instance with evidence of ongoing endothelial activation and injury may benefit from immunosuppressive protocols (such as corticosteroids and cyclophosphamide) employed in systemic vasculitis and more recently in small vessel vasculitis of the brain whilst this may not be the case for those with a monophasic disease course (Eleftheriou et al. 2009; Hutchinson et al. 2010).

In this thesis immunomagnetic bead extraction was used to detect and quantify CEC. Owing to CEC scarcity, this is the most widely used method for CEC enumeration based on a first step of enrichment by immunomagnetic separation followed by visual counting using a fluorescence microscope (Erdbuegger, Haubitz, & Woywodt 2006; Woywodt et al. 2006). Notably, the consensus definition of CECs and a standardized protocol by Woywodt et al. have led to good agreement among laboratories with regard to CEC levels in normal populations (Woywodt et al. 2006). This is also reflected herein in the little variation on CEC counts observed between different blinded observers counting CEC in the same patient sample. However, the identification and counting of CECs using fluorescence microscopy remains laborious and requires operator training. To date, flow cytometry measurement, the main alternative approach for CEC measurement, has been rather disappointing regarding accuracy of CEC quantification, although clearly is attractive since it would allow more detailed multi-parametric phenotyping of CECs. For instance the normal values for CECs derived by flow cytometry vary widely and in most cases, are higher (sometimes by orders of magnitude) than the values obtained using immunomagnetic bead extraction (Sabatier et al. 2008). Furthermore, there is no consensus regarding the combination of markers used to identify CEC. One other major issue is whether or not flow cytometry is sensitive enough to count cells in blood at low frequency. Clarke et al. recently reported on a study comparing immunomagnetic bead extraction and flow cytometry for the quantitative detection of CECs in systemic vasculitis (Clarke et al. 2008). Appropriate gating was identified as an important factor that needs further optimisation in flow cytometric protocols particularly in view of the fact that CECs often represent denucleated carcasses and parts of cells, hence not surprisingly will be smaller than HUVECs and appear in the mononuclear region of

FSC/SSC gating (Clarke et al. 2008). Additionally the use of lysing buffer may result in reduced recovery of CECs particularly in conditions such as systemic vasculitis where these cells are probably too vulnerable to survive after the flow cytometry preparation procedure (Clarke et al. 2008). Interestingly, Widemann et al. recently described a new hybrid assay for CEC detection that combines pre-enrichment of CD146+ circulating cells using magnetic nanoparticles and multiparametric flow cytometry analysis (Widemann et al. 2008). This promising approach combines a first step of enrichment that is compatible with CEC scarcity in peripheral blood, followed by flow cytometry of enriched cells that uses a multiparametric characterization of CEC and may be an automated counting alternative to microscopic observation (Widemann et al. 2008). It would be of considerable interest to adapt a similar technique and study the CEC phenotype in the circulation of children with AIS. This approach is additionally particularly attractive for large-scale clinical studies, to further establish the clinical value of CECs in vascular disorders.

Another important issue is whether the vascular bed origin of CECs varies according to the disease process and can be established using specific antigens. In addition to the structural heterogeneity, the endothelium displays different functions associated with the expression of specific surface antigens (Aird 2007; Chi et al. 2003; Sabatier et al. 2009). For instance CD36, a marker of microvasculature, has been used to attempt to document the anatomical origin of CEC at least in terms of size of vessel of origin (Lin et al. 2000; Sabatier et al. 2009; Solovey et al. 1997). In addition, a number of other constitutive or inducible antigens such as E-selectin, CD31, VCAM-1 or tissue factor have been used to identify pro-inflammatory and pro-coagulant activation of endothelial cells (Sabatier et al. 2009).

Assuming that CECs are representative of the vessel they derived from, analysis of their phenotype using new approaches of flow cytometric identification discussed above may provide important insights not only on their anatomical origin of these cells (cerebrovascular bed, peripheral endothelium etc.) but also on their biological role. The precise origin of CEC in this study however remains unclear.

This study has a number of limitations. It is possible that the intergroup differences may reflect the extent of endothelial injury relating to the bilateral multifocal arteriopathy seen in the group of children with AIS recurrence rather than recurrence risk per se. It will be of considerable interest to establish the specificity of these findings by comparison with patients with AIS without arteriopathy (who may have small vessel vasculitis of the brain as suggested by some authors), and in relation to other well-established risk factors such as cardiac disease. This study initially focused on children with AIS and cerebral arteriopathy specifically in order to inform the controversy surrounding the role of persistent vascular inflammation contributing to progression of arteriopathy in this group. Secondly, no cerebrovascular biopsies were performed to confirm persistent cerebral vasculitis, but this is reflective of current paediatric clinical practice where cerebro-arterial or other CNS biopsy is rarely undertaken (Benseler et al. 2006; Eleftheriou & Ganesan 2009). Of note, the time from recent AIS to evaluation was shorter in the group of children with recurrence compared to those with a single event. To control for this potentially confounding factor however a multivariable logistic regression was performed that showed CEC remained significantly associated with AIS recurrence after adjustment for this variable. Furthermore, longer duration of follow up is needed to ensure non recurrence of AIS in all patients studied. The



sample size of this study was small and restricted by patient numbers in this single centre cohort and referral bias cannot additionally be excluded. The small sample size also limits the interpretation of the logistic regression analysis and ROC analysis results. Additional criticism is that the ROC curve extends beyond the clinically relevant area of potential clinical interpretation while the AUC does not incorporate the pre-test probability of a disease. Lastly, further prospective studies to confirm or refute these observations are now needed and will require multicentre collaboration.

In conclusion, these data suggest that persistent endothelial injury could be an important mechanism underlying recurrent childhood AIS. The study of CECs provided a window to the activated endothelium in vivo and could lead to further understanding of stroke pathophysiology in children. Prospective validation of these findings is now required to firmly establish the role of these biomarkers as prognostic markers in childhood AIS and cerebral arteriopathy.

# 4 Circulating microparticles in children with arterial ischaemic stroke

## 4.1 Summary

**Background/objective:** Circulating microparticles (MP) are membrane derived vesicles released from cells (endothelial cells, platelets and leucocytes) in response to activation or apoptosis and are rich in procoagulant phospholipids, particularly phosphatidylserine (PS). Detailed profiling of these blood endothelial and other cellular markers may adumbrate the pathogenesis of arterial ischaemic stroke (AIS) in children or enable determination of the risk for AIS recurrence. In this chapter MP profiles in children with cerebral arteriopathy and AIS recurrence were compared to those children who had a single event.

**Methods:** Single centre cross-sectional study of 46 children with AIS and cerebral arteriopathy matched with 10 paediatric controls and 10 children with cerebral arteriovenous malformation (disease control group). AIS recurrence was defined as new acute neurological deficit with radiological evidence of further cerebral infarction. Microparticles were analysed using flow cytometry. Results were expressed as median and range.

**Results:** Total circulating Annexin V+ (PS rich) MPs were significantly higher in those with AIS recurrence compared to those children with no recurrence,  $p=0.002$ ; healthy controls,  $p=0.0001$  and children with cerebral AVM,  $p=0.0001$ . These MPs were of endothelial, platelet, neutrophil origin and monocytic origin. Tissue factor expressing MPs were elevated in children with recurrence compared to those with a single event,  $p=0.0040$ ; healthy control children,  $p=0.0001$ ; and children with cerebral AVM,  $p=0.0030$ . Monocytic MP also

expressed P-selectin glycoprotein ligand-1 (PSGL-1) that facilitates TF transfer onto activated platelets and thus further promotes thrombus formation.

**Conclusions:** Circulating MP profiles reflect distinct phenotypes of childhood AIS with cerebral arteriopathy and are markers of vascular pathology, platelet activation and thrombotic propensity relating to AIS recurrence.

## **4.2 Introduction**

In chapter 3 it was established that CEC non-invasively track endothelial injury associated with AIS recurrence and effectively differentiate between children with progressive and non-progressive disease courses. In acute AIS the mechanisms of brain ischaemia are however not only critically dependent on endothelial activation and vascular injury but also involve platelet activation, leukocyte migration and release of several soluble inflammatory mediators triggered during the ischaemic insult (Danton and Dietrich 2003; Dougherty et al. 1977). Recruitment of platelets subsequently results in activation of the coagulation cascade initiated mainly by tissue factor and leading to formation of fibrin (Lane et al. 1983). In physiological conditions regulatory mechanisms prevent thrombus from forming, but in pathological processes these mechanisms are overwhelmed and thromboses develop (Danton & Dietrich 2003; Lane et al. 1983). The relative contribution of these mechanisms to the dynamic and structural formation of a thrombus as the inciting event of AIS in children remains unclear however. Thus gaining some insight into the activation state of other cells besides endothelial cells that may play a pivotal role in disease pathogenesis and childhood AIS recurrence is crucial and may help us improve recurrence risk stratification strategies as well as develop more targeted therapies.

Cell activation and apoptosis is associated with the shedding of fragments from the plasma membrane termed microparticles (MPs) (Fadok et al. 2000; Fadok et al. 2001; Hugel et al. 2005; Morel et al. 2004). When cells are subjected to pro-inflammatory or pro-apoptotic stimuli there is a swift egress of negatively charged aminophospholipids to the outer leaflet of the plasma membrane, membrane budding and ultimately MP release (Hugel et al. 2004; Morel et al. 2006). MPs are thus cytosol-containing vesicles (100 nm to 1  $\mu$ m in diameter) comprised of bi-lipid membrane with an antigen distribution representative of the parent cell of origin, and enriched with procoagulant aminophospholipids, particularly phosphatidylserine (PS), in their exoplasmic leaflet (Dignat-George & Boulanger 2011; Leroyer et al. 2010; Morel, Toti, Hugel, & Freyssinet 2004). The protein content of MPs reflects that of the cell membrane from which they are released allowing us to differentiate MP of endothelial, platelet and leukocyte origin (Dignat-George & Boulanger 2011; Leroyer et al. 2010; Morel, Toti, Hugel, & Freyssinet 2004). Besides the cellular origin the nature of the trigger may also influence the number and phenotype of MPs. MPs have recently received attention as potent bioeffective vectors with multiple roles in inflammation, endothelial activation and thrombosis (Dignat-George & Boulanger 2011; Leroyer et al. 2010; Morel, Toti, Hugel, & Freyssinet 2004). The following sections provide an overview of MP formation, analytical methods and their pathophysiological roles in health and disease.

#### **4.2.1 Mechanisms of microparticle formation**

Although initially met with scepticism, the existence of a large number of extracellular vesicles including exosomes, activation- or apoptosis-induced microparticles and apoptotic bodies is well established (György et al. 2011). These structures are released via separate mechanisms and have different biological roles (György et al. 2011). The key features of cellular microparticles that distinguish them from these other microvesicles are summarised in table 4-1.

Our current understanding of the mechanism underlying the process of MP formation and release is based on a series of *in vitro* studies examining MP formation following activation of cells (endothelial, neutrophil, platelets and leukocytes) in response to a variety of stimuli (Dignat-George & Boulanger 2011). Combes et al. were amongst the first to describe the release of endothelial microparticles (EMP) from HUVEC in response to TNF- $\alpha$  stimulation (Combes et al. 1999).

Under physiological conditions aminophospholipids such as phosphatidylserine (PS) and phosphatidyl-ethanolamine (PE) are specifically enriched in the inner leaflet, whereas phosphatidylcholine (PC) and sphingomyelin are segregated in the external one (Hugel et al. 2005). The maintenance of lipid asymmetry relies on an adenosine triphosphate (ATP)-dependent transporter, the aminophospholipid translocase, governing inward (flip) or outward (flop) translocation that specifically and rapidly conveys PS and PE from the outer to the inner leaflet and that is inhibited by calcium ions (Hugel et al. 2005; Seigneuret et al. 1984; Zwaal and Schroit 1997). Membrane remodelling and disruption of the basal

membrane lipid asymmetry is a central event to cells undergoing activation or apoptosis (Hugel et al. 2005; Seigneuret, Zachowski, Hermann, & Devaux 1984; Zwaal & Schroit 1997). With an aim of providing a pro-coagulant surface transporters facilitate the rapid egress of PS at the outer leaflet in cells undergoing apoptosis (Hugel et al. 2005; Seigneuret, Zachowski, Hermann, & Devaux 1984; Zwaal & Schroit 1997). Translocators include members of the floppase family (transporters including the ATP-binding cassette transporter A1, ABCA1), and the calcium-dependent scramblase that facilitate bidirectional movement between membrane leaflets (Morel et al. 2010). Cell stimulation results in increased cytosolic  $[Ca^{2+}]$  concentration and the rapid translocation of aminophospholipids to the outer leaflet, while flippase activity is reduced and there is an increased floppase/ scramblase activity favouring phospholipid exchanges between the two leaflets (Morel, Toti, Jesel, & Freyssinet 2010). Surface tension and membrane budding ultimately lead into MP shedding (Morel, Toti, Jesel, & Freyssinet 2010).

Lipid rafts may also have a role in membrane responses to activation and apoptosis (Del Conde et al. 2005b; Kunzelmann-Marche et al. 2002; Simons and Toomre 2000). Rafts are functional transbilayer domains that serve as organizing centres for the assembly of particular proteins and lipids as well as influence membrane fluidicity and membrane protein trafficking (Simons & Toomre 2000). PS exposure is significantly reduced after raft disruption and proteins with strong cytoskeleton interactions are not represented as frequently in MPs (Davizon et al. 2010; Del Conde et al. 2005b; Kunzelmann-Marche et al. 2002).

Furthermore, a number of studies have recently explored the molecular mechanisms governing the release of endothelial derived MP (EMP) in particular. Sapet et al. used gene profiling analysis and identified an original pathway induced by thrombin, depending on nuclear factor-kB activation, and involving the Rho-kinase ROCK-II activation by caspase 2 in the absence of cell death (Sapet et al. 2006). The process involves 2 steps: a first phase that follows thrombin binding to its receptor protease-activated receptor-1 (PAR-1) and a second phase that relies on transcriptional events mediated by thrombin and involving TRAIL/Apo2L, a cytokine belonging to the TNF- $\alpha$  superfamily (Sapet et al. 2006; Simoncini et al. 2009). Gene profiling studies additionally identified interleukin-1 and interleukin-1Ra, as additional players of thrombin-induced EMP (Leroyer et al. 2010). Thrombin stimulation of human microvascular endothelial cells (HMEC-1) resulted in an increase in IL-1R1 expression, a concomitant decrease in IL-1Ra and a low secretion of IL-1 (Leroyer et al. 2010). Thrombin-induced EMP release was inhibited by specifically silencing of IL-1R1 or IL-1 (Leroyer et al. 2010). Furthermore, the engagement of IL-1R1 resulted in the recruitment of adaptor proteins TRAF6 (TNF receptor associated protein 6) and IRAQ1 that activated a signalling pathway leading to the amplification of EMP release by thrombin (Leroyer et al. 2010). Lastly, another study showed that p38 mitogen-activated protein kinase is a critical pathway in the production of EMP (Curtis et al. 2009).

	<b>Exosomes</b>	<b>Microparticles</b>	<b>Apoptotic bodies</b>
<b>Size range</b>	Approximately 50–100 nm	100–1,000 nm	1–5 µm
<b>Mechanism of generation</b>	By exocytosis of multi-vesicular bodies	By budding/blebbing of the plasma membrane	By release from blebs of cells undergoing apoptosis
<b>Isolation</b>	Differential centrifugation and sucrose gradient ultracentrifugation, 100,000–200,000g, vesicle density is 1.13–1.19 g/mL	Differential centrifugation 15,000–20,000g	Established protocols are essentially lacking; most studies use co-culture with apoptotic cells instead of isolating apoptotic bodies
<b>Detection</b>	Electron microscopy, western blotting, mass spectrometry, flow cytometry	Flow cytometry	Flow cytometry
<b>Best characterised cellular sources</b>	Immune cells and tumors	Platelets, red and white blood cells and endothelial cells	Cell lines
<b>Markers</b>	Annexin V binding, CD63, CD81, CD9, LAMP1 and TSG101	Annexin V binding, tissue factor and cell-specific markers	Annexin V binding, DNA content
<b>Lipid composition</b>	Cheramides, cholesterol	Cholesterol	Not known
<b>Intracellular storage</b>	Yes	No	No

*Table 4-1: Key features of microvesicles. Adapted from Gyorgy et al. (2011)*



#### ***4.2.2 Current methods to identify and enumerate microparticles***

Several anticoagulants have been used to collect blood for MP analysis (Yuana et al. 2011). Sodium citrate with a concentration of 3.2% or 3.8% is the most widely used anticoagulant currently (Jy et al. 2004). Other anticoagulants that have been used include ethylenediaminetetraacetic acid (EDTA) a strong chelator of calcium ions, and heparin which will preserve extracellular calcium (Aras et al. 2004; Ueba et al. 2008; Yuana, Bertina, & Osanto 2011). However, levels of annexin V-positive MPs are not directly comparable in blood samples collected in EDTA to those collected in sodium citrate (Connor et al. 2009; Yuana, Bertina, & Osanto 2011). In addition, platelets need to be removed from the plasma when processing samples for MP analysis in order to avoid platelet activation leading to inadvertent production of MPs (Jy, Horstman, Jimenez, & Ahn 2004). Hence MP are usually analysed in platelet poor plasma samples (Jy, Horstman, Jimenez, & Ahn 2004). Furthermore, cold activation of citrated blood samples has been reported to result in falsely elevated levels of (activated) factor VII (FVII) and factor VIII (FVIII) (Favaloro et al. 2004). Therefore, the temperature is generally kept at room temperature (20–25°C) for centrifuging citrated blood (Jy, Horstman, Jimenez, & Ahn 2004; Yuana, Bertina, & Osanto 2011). Furthermore, during centrifugation the use of brakes should be avoided and plasma should be carefully collected without disturbing the platelet layer to prevent remixing of the plasma with the platelets (Yuana, Bertina, & Osanto 2011). Ideally, plasma is prepared immediately after blood collection however from the practical point of view time samples may need to be stored prior to analysis (Jy, Horstman, Jimenez, & Ahn 2004; Yuana, Bertina, & Osanto 2011). During storage blood samples should be maintained at room temperature while

mechanical agitation is avoided for reasons discussed above (Jy, Horstman, Jimenez, & Ahn 2004; Yuana, Bertina, & Osanto 2011).

Flow cytometry has been widely used to quantify MP in clinical studies although a number of limitations are recognized (Jy, Horstman, Jimenez, & Ahn 2004; Kim et al. 2002; Lacroix et al. 2010a; Yuana, Bertina, & Osanto 2011). Certain properties of MP are used to identify and enumerate MP: MPs are vesicles found in platelet-poor plasma, with size ranging for 0.1–1  $\mu\text{m}$  and expressing specific antigens of the cell they derived from (Brogan et al. 2004; Leroyer et al. 2010; Morel, Toti, Hugel, & Freyssinet 2004). Generally, the use of beads as an internal standard provides standardized conditions for accurate numeration of circulating MPs (Aras et al. 2004; Brogan et al. 2004; Kim et al. 2002). These are calibrated polypropylene latex beads that are fluorescent across a range of excitation and emission wavelengths. Counting beads are processed as a sample at the same flow cytometry settings/conditions used for the samples (Brogan et al. 2004; Clarke et al. 2010; Hong et al. 2012; Shet et al. 2003). The flow cytometer counts the number of beads and/or MPs in the sample until the set acquisition time is reached and with these numbers, the concentration of MPs in plasma can be calculated (Brogan et al. 2004; Clarke et al. 2010; Hong et al. 2012; Shet et al. 2003). As far as the flow cytometry settings are concerned, the MP population is defined by using beads of 1  $\mu\text{m}$  diameter (Aras et al. 2004; Hong et al. 2012; Kim et al. 2002). These calibrated beads define the upper limit of the MP population and help discriminate the MP population from platelets (Aras et al. 2004; Hong et al. 2012; Kim et al. 2002). Other investigators have more recently used a mixture of fluorescent beads with diameters of 0.5, 0.9, and 3  $\mu\text{m}$  (Megamix beads) to cover the MP (0.5 and 0.9  $\mu\text{m}$ ) and platelet populations (0.9 and 3  $\mu\text{m}$ ) ranges (Lacroix et al. 2010b). The majority of MPs expose PS therefore

fluorescently labelled annexin V that binds to PS is commonly used to measure the total number of MPs with flow cytometry (Brogan et al. 2004; Clarke et al. 2010; Hong et al. 2012). Of note the binding of annexin V to MPs is influenced by the calcium concentration and the membrane PS content (Yuana, Bertina, & Osanto 2011). In addition, the cellular origin of MP can be assessed by staining MPs with relevant monoclonal antibodies (Brogan et al. 2004; Clarke et al. 2010; Hong et al. 2012). Considering the heterogeneity of MPs, an ideal marker should insure specificity toward one cell type and be expressed whatever the potential mechanisms that trigger vesiculation (Brogan et al. 2004; Clarke et al. 2010; Hong et al. 2012).

Notably, pre-analytical conditions still represent an important source of variability in the analysis of MP (Robert et al. 2009; Yuana, Bertina, & Osanto 2011). The type of instrument, instrument settings, and resolution also influence flow cytometric measurements (Robert et al. 2009; Yuana, Bertina, & Osanto 2011). Recently, the Vascular Biology group of the Scientific and Standardisation Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) proposed the use of calibrated beads to standardise the enumeration of MPs  $>0.5 \mu\text{m}$  by using flow cytometry (Robert et al. 2009). Forty laboratories from 14 countries participated in the study using several types of flow cytometer such as FACSCalibur, FACSCanto II, and LSRII (all from Becton Dickinson, Le Pont de Claix Cedex, France) and EPICS XL, Gallios and FC500 (all from Beckman Coulter, Paris, France) (Robert et al. 2009). In addition Lacroix et al. have recently studied the relative impact of different preanalytical parameters on circulating MP and identified the pre-analytical processing of the samples from healthy donors, agitation during transportation, time-delay,

and centrifugation conditions were identified as the three major parameters that have the strongest effects on MP measurement (Lacroix et al. 2012). At present, standardisation of pre-analytical and analytical methods for the measurement of MPs remains challenging (Robert et al. 2009; Yuana, Bertina, & Osanto 2011).

#### ***4.2.3 Non flow cytometric methods used to evaluate microparticles***

Electron and fluorescence confocal laser scan microscopy have also been used to evaluate MP populations (Yuana, Bertina, & Osanto 2011). Electron microscopy (EM) has been employed to study the morphology and membrane composition of MPs (Bernimoulin et al. 2009; Heijnen et al. 1999). In addition staining with immunogold-labelled antibodies can provide information on the antigenic composition of isolated MPs (Bernimoulin et al. 2009; Heijnen et al. 1999). Using EM, Bernimoulin et al. for instance were able to show that MPs derived from THP-1 cells contain cytoskeletal proteins organised into a complex cytoskeletal network (Bernimoulin et al. 2009). Confocal laser microscopy permits identification of subsets of MPs based on their antigen expression (Yuana, Bertina, & Osanto 2011). Dale et al. employed confocal laser microscopy to characterise platelet MPs (PMPs) (Dale et al. 2005). These PMPs were 0.3–0.5  $\mu\text{m}$  in diameter and positive for glycoprotein IIb/IIIa, glycoprotein Ib, CD9, and PS, but negative for fibrinogen and thrombospondin (Dale, Remenyi, & Friese 2005). EM and fluorescence confocal laser scan microscopy in general give information on morphological features of MPs (e.g. size, membrane structure, cytoskeleton) but the operation of both methods is laborious and they are not suitable for direct detection and quantification of MPs in plasma for clinical studies (Yuana, Bertina, &

Osanto 2011). For capture-based assays, wells are coated with an antibody (usually annexin V) that specifically binds a subset of MPs from plasma (Nomura et al. 2009; Ueba et al. 2008). Captured MPs can then be quantified/ characterised by using a second antibody or by exploring the functional properties of the captured microparticles, for instance their procoagulant activity (Nomura et al. 2009; Ueba et al. 2008). At present however there is limited experience with this type of approach. Mass spectrometry (MS)-based proteomic analysis provides an opportunity to characterise the protein composition of MPs (Miguet et al. 2007; Yuana, Bertina, & Osanto 2011). Generally this method requires extraction, separation, trypsin digestion, MS analysis, and identification of proteins and is not yet widely used (Miguet et al. 2007; Yuana, Bertina, & Osanto 2011).

#### ***4.2.4 Microparticles as important thrombotic protagonists***

A very important aspect of the role of MP in health and disease is their prothrombotic potential (Morel et al. 2006; Piccin, Murphy, & Smith 2007). MPs fulfill a haemostatic function under physiological conditions through one of their main defining characteristics, the exposure of the anionic aminophospholipid, PS in their surface that provides a platform for the assembly and activation of coagulation factors (Piccin, Murphy, & Smith 2007). Scott syndrome is a well described inherited haematological disorder characterized by impaired transmembrane migration of procoagulant PS and defective MP formation that is associated with haemorrhagic complications, thus highlighting the important role of MPs in physiological haemostasis (Toti et al. 1996). MPs are protective in case of bleeding but their dramatic release could favour thrombotic complications in the arterial or venous circulation (Morel et al. 2006; Piccin, Murphy, & Smith 2007). An excess of production MPs could be

involved in shifting the homeostatic balance between pro- and anticoagulant forces in favor of coagulation (Piccin, Murphy, & Smith 2007). This could be mediated through PS exposure providing a surface for the amplification of coagulation enzymes to induce a pro-thrombotic state (Holme et al. 1998; Sabatier et al. 2002). In addition some MPs carry tissue factor (TF), the initiator of the extrinsic coagulation pathway (Zwicker et al. 2011). MPs notably constitute the main reservoir of blood-borne TF released from monocytes, endothelial cells or vascular smooth cells and fibroblasts (Giesen et al. 1999; Morel et al. 2006). Cytokines, thrombin, low shear stress, oxidative stress, hyperglycemia, dyslipidemia, complement activation amongst others induce procoagulant PS exposure and TF induction on MPs (Cermak et al. 1993b; Gûrlach et al. 2000; Muhlfelder et al. 1979; Penn et al. 2000). TF acts synergistically with PS when expressed by the membrane of stimulated cells to initiate coagulation (Mackman et al. 2007; Morel et al. 2006). The actual composition of the phospholipid surface on MPs surrounding the TF/FVIIa complex is an essential determinant of its activity, with anionic phospholipids greatly potentiating the TF cofactor function even if it is marginally expressed (Bach 2006).

This procoagulant potential of MP was first demonstrated by Combes et al. that observed a reduction of the clotting time of normal plasma incubated with increasing amounts of endothelial derived MP released in vitro (Combes et al. 1999). The thrombogenic capacity of MP was then confirmed by the demonstration that MP triggered TF-dependent thrombin formation in vitro and thrombus formation in vivo (Del Conde et al. 2005a; Falati et al. 2002; Falati et al. 2003). Experimental models have also shown that when thrombosis begins there is accumulation of leukocyte-derived MPs harbouring TF activities whereas the level

of platelet-derived MPs mostly depends on the extent of platelet stimulation (Falati et al. 2002; Falati et al. 2003; Gross et al. 2005; Morel et al. 2009; Ramacciotti et al. 2009). Notably, Ramacciotti et al. demonstrated significant differences in the involvement of leukocyte and platelet MPs in the thrombogenic process (Ramacciotti et al. 2009). In an experimental mouse model of venous thrombosis, circulating leukocyte-derived MP levels were negatively correlated with the thrombus weight, suggesting their early incorporation within the growing thrombus while platelet-derived MPs were positively correlated with the thrombus growth (Ramacciotti et al. 2009). Interestingly Hrachovinova et al. demonstrated that the infusion of soluble P-selectin results in the generation of leukocyte derived MPs that are then able to correct the bleeding diathesis in a mouse model of haemophilia A (Hrachovinova et al. 2003). Other mechanisms triggered by P-selectin that induce a prothrombotic state include: promotion of PS exposure and TF expression on monocytes (Celi et al. 1994; Celi et al. 2004); transfer of TF sorted from rafts into monocyte derived MPs that then get delivered to activated platelets (Del Conde et al. 2005a); and generation of monocyte MPs enriched in lymphocyte function-associated-antigen-1(LFA-1;CD11a/CD18) that enable interactions between leukocytes and endothelial cells (Bernimoulin et al. 2009). In addition, several studies have shown that the recruitment at the edge of thrombus of leukocytes and leukocyte MPs is dependant on P selectin and P-selectin glycoprotein ligand-1 interactions (Falati et al. 2002; Falati et al. 2003; Thomas et al. 2009). Moreover, TF-positive MP can bind to other cell types, such as monocytes, and transfer bioactive TF (Rauch et al. 2000; Sabatier et al. 2002). Mesri et al. have demonstrated up-regulation of circulating leukocyte MPs in vivo and induction of stress signalling pathways in endothelial cells, resulting in to increased TF activity (Mesri and Altieri 1999). Additionally monocyte-

derived-MPs up-regulated the expression of active TF by endothelial cells and induced a rapid expression of von Willebrand factor at the endothelial cell surface (Essayagh et al. 2007). In vitro experiments demonstrated that these two major cell responses are under the control of reactive oxygen species (ROS) delivered by MPs (Essayagh et al. 2007).

In parallel to this leukocyte-platelet-endothelium interaction that play a major role in thrombus generation, other cellular lineages may contribute to MP-driven blood coagulation. In traumatic brain injury, Morel et al. have demonstrated an increased generation of procoagulant MPs in the cerebrospinal fluid that may reflect neuronal apoptosis (Morel et al. 2008a). In addition, a number of studies have shown that erythrocyte derived MPs correlate with the extent of blood coagulation activation (Mfonkeu et al. 2010; Van Beers et al. 2009). Therefore by exposing PS and TF, MP contribute significantly to a procoagulant potential and could be important thrombotic protagonists in the context of several vascular disorders. Notably, a series of clinical studies have confirmed the presence of procoagulant MP in atherosclerosis, sickle cell anaemia and in patients with acute coronary syndrome (Chironi et al. 2006; Koga et al. 2005; Shet et al. 2003; Werner et al. 2006).

#### ***4.2.5 Microparticles: inflammatory and anti-inflammatory effects***

Several studies have demonstrated the proinflammatory effects of MPs on the endothelium. MPs may affect the endothelial phenotype via a transcellular platelet-derived microparticles (PMP)-mediated delivery of arachidonic acid to endothelial cells and the concomitant expression of cyclooxygenase type 2 (Barry et al. 1997). In addition, MPs have been shown



to induce the release of proinflammatory cytokines, including IL-6 or MCP-1 from endothelial cells and stimulate the up regulation of ICAM-1, VCAM-1 and E-selectin on the surface of endothelial cells (Barry et al.1997; Mesri and Altieri 1998;Mesri & Altieri 1999; Müller et al. 2009; Nomura et al. 2001). More recently, circulating PMPs were shown to serve as a transcellular delivery system for RANTES, triggering monocyte arrest to inflamed and atherosclerotic endothelium (Mause et al. 2005). In addition monocyte-derived MPs have been shown to secrete IL-1 $\beta$  (MacKenzie et al. 2001). Furthermore a growing number of clinical studies support the proinflammatory character of MPs (Morel, Toti, Jesel, & Freyssinet 2010). In the synovial fluid of patients with rheumatoid arthritis (RA), MPs from monocytes, granulocytes and more recently platelets have been identified as modulators of chemokine and cytokine production by fibroblast like synoviocytes (FLS) (Berckmans et al. 2002;Boilard et al. 2010). Cerebral malaria is another example of the MP pro-inflammatory potential with experimental data having established the important role of erythrocytes-derived MPs during malaria infection (Faille et al. 2009). In sepsis, NO and bacterial elements are responsible for the generation of PMP that have an active role in vascular signaling (Gambim et al. 2007; Morel, Toti, Jesel, & Freyssinet 2010).

Of note, a series of studies have specifically explored the role of neutrophil microparticles (NMP) as inflammatory mediators in vascular disorders (Dalli et al. 2008; Daniel et al. 2006; Gasser and Schifferli 2004; Hong et al. 2012). In adults with systemic vasculitis, Daniel et al. observed increased plasma neutrophil microparticles although the pathogenic potential of these were not investigated (Daniel et al. 2006). Subsequently, Hong et al. also showed an increase in neutrophil microparticles in the plasma of children with antineutrophil

cytoplasmic antibody (ANCA) associated vasculitis compared with that in healthy controls or those with inactive vasculitis (Hong et al. 2012). In the same study it was demonstrated that ANCA induced the release of neutrophil microparticles from primed neutrophils (Hong et al. 2012). These microparticles expressed a variety of markers, bound endothelial cells via a CD18-mediated mechanism and induced an increase in endothelial ICAM-1 expression, production of endothelial reactive oxygen species, and release of endothelial IL-6 and IL-8 (Hong et al. 2012).

Interestingly, Gasser et al. also showed that NMPs could exert anti-inflammatory influence on monocytes by suppressing proinflammatory cytokine production and increasing TGF- $\beta$  secretion (Gasser & Schifferli 2004). Dalli et al. also proposed that certain NMP subpopulations may exert an anti-inflammatory role (Dalli et al. 2008). They generated NMPs by coculture of neutrophils with human umbilical vein endothelial cells (HUVECs) in the presence of fMLP (Dalli et al. 2008). The NMPs derived from the neutrophils adherent to HUVECs were rich in the anti-inflammatory protein annexin 1, and demonstrated the ability to inhibit further neutrophil adhesion to endothelium (Dalli et al. 2008). Importantly, these anti-inflammatory NMPs represented the nonadherent NMP population generated in this model, and it is possible that proinflammatory NMPs were removed by binding HUVECs and would not have been included in the NMP analysis and could explain the apparent conflicting results between these studies of NMP (Hong et al. 2012).

#### **4.2.6 Microparticles and angiogenesis**

MPs have also been shown to play a major role in angiogenesis either through MP production of pro-angiogenic factors or in some cases by decreasing the production of anti-angiogenic factors (Agouni et al. 2007; Benameur et al. 2010a; Benameur et al. 2010b; Kim et al. 2004; Martinez et al. 2005; Martinez et al. 2006; Mause et al. 2010; Soleti et al. 2009). Recently, Benameur et al. have shown that circulating MPs from peroxisome proliferator-activated receptor- $\alpha$ -wildtype mice, but not from knockout mice, induce the formation of capillary-like structures of endothelial cells (Benameur et al. 2010b). In addition, Kim et al. demonstrated that PMP can stimulate human endothelial cells to proliferate, migrate and form tubes essential for angiogenesis, *in vitro* (Kim et al. 2004). Both protein and lipid growth factors were involved but it seemed that the effects of the latter were greater (Kim et al. 2004). Moreover, MPs generated from either activated or apoptotic T-lymphocytes express the morphogen Sonic hedgehog (MPs<sup>Shh+</sup>) on their surface and promote angiogenesis (Benameur et al. 2010a; Benameur et al. 2010b; Martinez, Tesse, Zobairi, & Andriantsitohaina 2005; Martinez et al. 2006; Soleti et al. 2009). *In vivo* treatment of hind limb ischaemic mice with MPs<sup>Shh+</sup> enhanced the neovascularization in the ischemic leg (Benameur et al. 2010a). Furthermore, several reports show that MPs from endothelial cells can affect endothelial progenitor cell (EPC) function and angiogenesis (Benameur et al. 2010b; Mause et al. 2010). Mause et al. observed that treatment of EPC with PMP increased expression of mature endothelial cell markers on the progenitor cells and promoted both endothelial cell adhesion and paracrine activity, leading to improved endothelial healing (Mause et al. 2010). Thus these reports suggest that MPs from ischaemic tissues could act as endogenous survival signals for vascular repair (Agouni et al. 2007; Benameur

et al. 2010a; Benameur et al. 2010b; Kim et al. 2004; Martinez, Tesse, Zobairi, & Andriantsitohaina 2005; Martinez et al. 2006; Mause et al. 2010; Soleti et al. 2009).

#### ***4.2.7 Microparticles in relation to vascular injury and endothelial dysfunction***

MPs are considered reliable markers of cell damage detectable in biologic fluids (Morel et al. 2006; Piccin, Murphy, & Smith 2007). It has been previously demonstrated that it is possible to monitor endothelial injury in systemic vascular disorders by the detection of circulating EMPs expressing E-selectin, or CD105 (Brogan et al. 2004; Clarke et al. 2010; Daniel et al. 2006; Erdbruegger et al. 2008). In children with systemic vasculitis Brogan et al. demonstrated an elevation in circulating E-selectin and CD105 expressing EMPs as well as CD42a (platelet) derived MP during active disease (Brogan et al. 2004). In addition, Daniel et al. showed an elevation in neutrophil microparticles (NMP) expressing CD66b and platelet MPs in adult patients with anti-neutrophil cytoplasmic antibody associated vasculitis (AAV) during active disease compared to controls (Daniel et al. 2006). NMP were also shown to decline in numbers with therapy and in disease remission (Daniel et al. 2006). Erdbruegger et al. examined EMP in adults with AAV confirming an increase in EMP and platelet MP during active disease (Erdbruegger et al. 2008). Notably, Clarke et al. recently studied MP profiles in children with systemic vasculitis at various disease stages establishing circulating MP as a robust biomarker of endothelial injury and platelet activation in this disease (Clarke et al. 2010).

MPs have also been studied in several other cardiovascular diseases (Bakouboula et al. 2008; Koga et al. 2005; Preston et al. 2003; Yuana, Bertina, & Osanto 2011). Current lines of evidence suggest that increased circulating levels of EMP for instance are associated with coronary artery disease (CAD) and vascular risk factors such as arterial hypertension, diabetes, hyperlipidaemia and others (Bakouboula et al. 2008; Koga et al. 2005; Preston et al. 2003; Yuana, Bertina, & Osanto 2011). Levels of EMP are also related to poor clinical cardiovascular outcome in these subjects (Bakouboula et al. 2008; Koga et al. 2005; Preston et al. 2003; Yuana, Bertina, & Osanto 2011). Circulating MPs could additionally be an important regulator of the vascular tone. Platelet microparticles have been shown to act as a cellular source of thromboxane A<sub>2</sub> and enhance arachidonic acid–induced contractions in the rabbit aorta (Pfister 2004). Furthermore, Martin et al. demonstrated that MPs from apoptotic T lymphocytes impair endothelial function and regulate endothelial protein expression (Martin et al. 2004). Tesse et al. observed that circulating MP from pre-eclamptic women induce vascular hyperactivity in vessels from pregnant mice through an overproduction of NO (Tesse et al. 2007). Lastly, MP from apoptotic smooth muscle cells (SMCs) have been shown to induce endothelial dysfunction by diminishing NO production in mouse aorta (Essayagh et al. 2005)

#### ***4.2.8 Microparticles in cerebrovascular diseases***

There are limited but promising data relating to MP profiles in cerebrovascular diseases (Jung et al. 2009; Shirafuji et al. 2008; Simak et al. 2006). In adults Jung et al. prospectively examined 348 consecutive patients with cerebrovascular disease: 73 patients with acute

stroke and 275 patients with vascular risk factors but no stroke events (Jung et al. 2009). Recent ischaemic episodes were found to be more strongly associated with greater E-selectin (CD62E+) endothelial derived MP levels (EMP) than with levels of other phenotypes (Jung et al. 2009). Increased National Institutes of Health Stroke Scale scores and infarct volumes in acute stroke patients were significantly associated with greater CD62E+EMP levels (Jung et al. 2009). The ratio of CD62E+ to CD31+/CD42b- or CD31+/AV+ EMP level significantly discriminated extra cranial and intracranial arterial stenosis (Jung et al. 2009). Thus circulating EMP phenotypic profiles could reflect distinct phenotypes of cerebrovascular disease, and provide additional information on anatomic origin and pathogenetic mechanisms (Jung et al. 2009). Simak et al. demonstrated significantly higher PS positive EMP counts during acute ischaemic stroke while EMP counts correlated significantly with brain lesion volume and discharge clinical outcomes (Simak et al. 2006). Lastly, Shirafuji et al. demonstrated elevated levels of platelet derived MP in patients with chronic cerebral infarction regardless of the use of anti-platelet therapy (Shirafuji, Hamaguchi, & Kanda 2008).

To date, however, circulating MP profiles in children with AIS and cerebral arteriopathy have not been studied. It would be of considerable interest to establish whether children with AIS recurrence have altered MP signatures indicative of platelet and endothelial activation. Moreover, these MP could exert important prothrombotic influence in those with progressive AIS due to PS and tissue factor expression if present in the circulation of these children. In this chapter MP are examined in this context.

## **4.3 Aims**

To investigate whether MP profiles differ between children with cerebral arteriopathy and AIS recurrence compared to those with a single event, as a consequence of neutrophil, monocyte and platelet activation in children with AIS recurrence.

## **4.4 Methods**

### ***4.4.1 Patients***

This was a cross sectional study of children > 28 days old presenting to Great Ormond Street Hospital with AIS. For inclusion and exclusion criteria, patient group classification, healthy controls and disease controls see chapter 2.

### ***4.4.2 Isolation of microparticles from platelet poor plasma***

One to 4 mls of whole blood was collected into bottles containing 3.2% trisodium citrate (Becton Dickinson). Platelet poor plasma (PPP) was obtained by immediate centrifugation of the whole blood at 5000g for 5 minutes twice. Plasma was then stored at  $-80^{\circ}$  C for batch analysis. Prior to analysis PPP was thawed rapidly in a  $37^{\circ}$ C water bath. Exact volumes of plasma (200-400 $\mu$ l) were then centrifuged at 17000 g for 60 minutes and the supernatant decanted to obtain the microparticle pellet. The MP were then reconstituted in An V binding buffer (BD PharMingen, Oxford, United Kingdom), divided into 40  $\mu$ l aliquots and plated onto the wells of a 96 well U-bottomed plate prior to staining with Annexin V and other monoclonal antibodies.

#### ***4.4.3 Labelling of microparticles with annexin V and monoclonal antibodies***

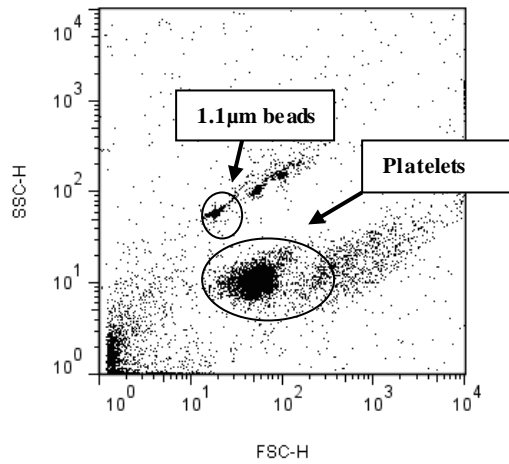
The labelling and quantification of MP was achieved as follows: 5 µl of a 1 in 5 dilution Annexin V conjugated with fluorescein isothiocyanate (FITC; BD Pharmingen) or - phycoerythrin (PE; BD Pharmingen) or PERCP-Cy5.5 (BD, Pharmingen) was added to every well in a 96 well U bottomed plate. For measuring endothelial derived MPs (EMPs) samples were additionally incubated with mouse (PE)-labeled anti-human CD62e (Clone 68-5H11, BD Pharmingen), mouse (PE)-labeled anti-human CD31 (clone L133.1, BD Pharmingen), mouse (PE) labeled anti human CD54 (clone LB-2, BD pharmingen) and mouse (PE) labeled anti human CD 106 (clone 51-10C9, BD Pharmingen). In platelet derived MPs (PMPs) expression of the platelet activation marker P selectin was assessed with staining with mouse (PE) labeled anti human CD62P (clone AC1.2, BD Pharmingen). For neutrophil derived MPs (NMPs) MP were stained with mouse (PE)-labeled anti-human CD11b activation epitope (clone CBRM1/5, Biolegend, UK). All samples were additionally stained with the constitutively expressed platelet marker CD42a (mouse IgG1 anti-human CD42a-PERCP, BD Pharmingen) to exclude a platelet origin for these MPs as well as the relevant isotype controls (as per manufacturer recommendation). To assess P-selectin glycoprotein ligand 1 (PSGL-1) and tissue factor (TF) expression on monocyte MPs, samples stained with mouse (Cy5)-labeled anti-human CD14 (clone 61D3, AbD Serotec) were additionally incubated with mouse (FITC)-labeled anti-human TF (clone VD8, American Diagnostica, USA) and mouse (PE)-labeled anti human PSGL-1 (clone KPL1, BD Pharmingen). All antibodies were used at final dilutions listed in table 2-1. MPs were incubated with the labeled antibodies and annexin V for 20 minutes at room temperature in



the dark with gentle shaking. The incubation was then terminated by adding 200  $\mu$ l of annexin V buffer to each well, and the samples transferred to small FACS tubes prior to flow cytometry. The plate plans used for MP detection are shown in appendix 3.

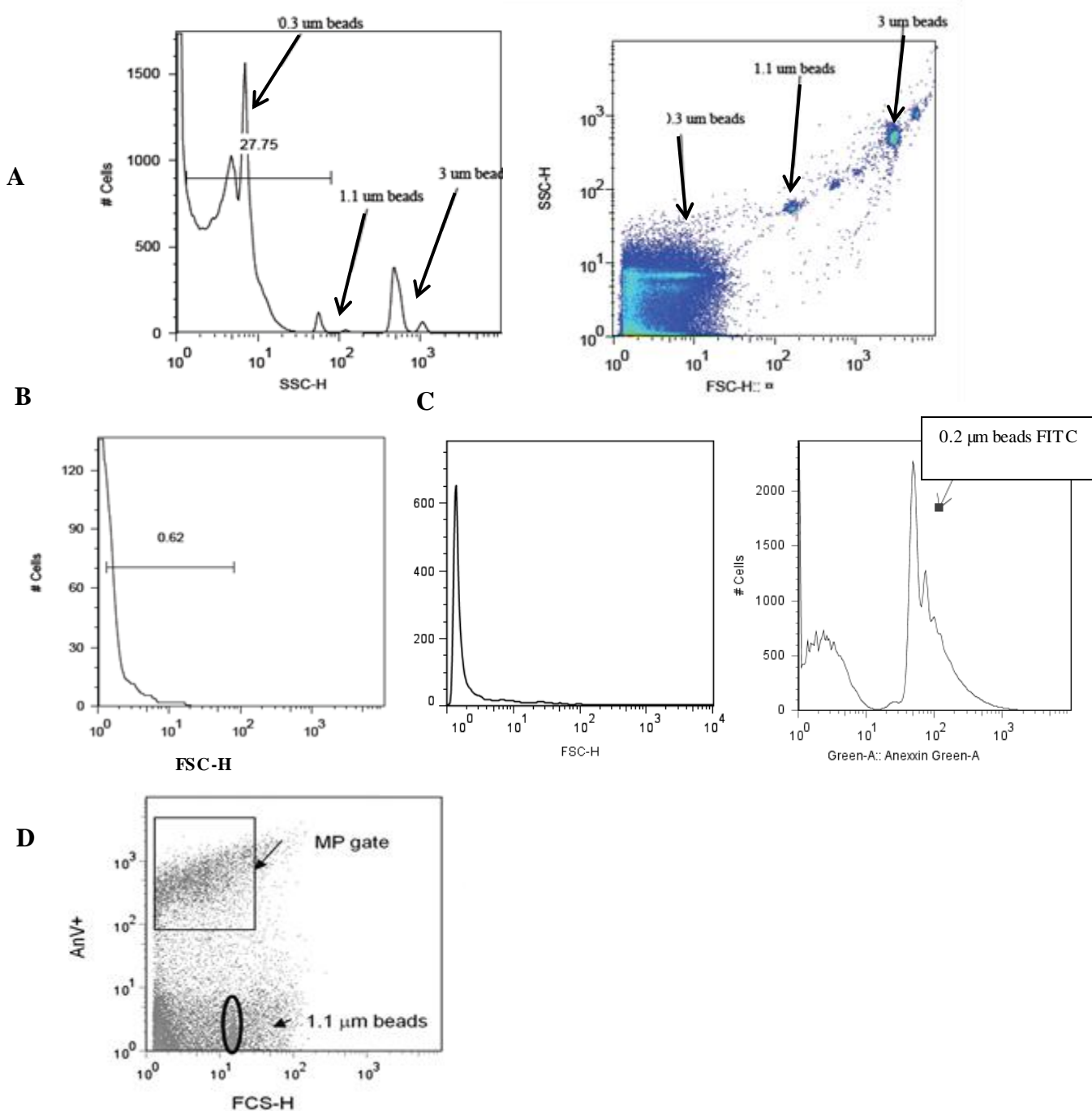
#### ***4.4.4 Optimization of gating strategy for flow cytometric analysis of microparticles***

To establish the limits of detection and optimize the gating strategy standard latex beads size of 0.2  $\mu$ m, 0.3  $\mu$ m, 1.1  $\mu$ m and 3  $\mu$ m (Sigma) were used. Of note these beads provide an approximate estimate of size and may not be directly comparable to MP size as they have different optical properties. As shown in figure 4-1, 1.1  $\mu$ m beads lie within a similar FSC location as platelets when whole blood was examined and thus could be used to set the upper boundary of the FSC region of interest and gate for MP population to < 1.1  $\mu$ m. In order to establish the lower limits of detection 0.2 and 0.3  $\mu$ m beads and sterile filter water were examined. The background noise detected using sterile filter water using a 0.2  $\mu$ m pore size filter was considered electrical noise (figure 4-2). Using this threshold the 0.2 and 0.3  $\mu$ m beads were examined. As depicted in figure 4-2 the 0.3  $\mu$ m beads could be distinguished from noise but not the 0.2  $\mu$ m beads. Therefore as MP cannot be entirely distinguished from background electrical noise based on their FSC and SSC properties alone, emphasizing the importance of using AnV (binding to PS) labeled with FITC, PE or PERCP as a constitutive MP marker. Figure 4-2 shows that 0.2  $\mu$ m FITC labeled beads are indeed detectable on the FL1 channel. The MP population was therefore in this study defined as AnV+ and size < 1.1 $\mu$ m (figure 4-2D).



**Figure 4-1: Gating strategy.**

*Figure 4-1 shows that 1.1 μm beads lie within a similar FSC location as platelets when whole blood was examined and thus could be used to set the upper boundary of the FSC region of interest and gate for MP population to < 1.1 μm.*

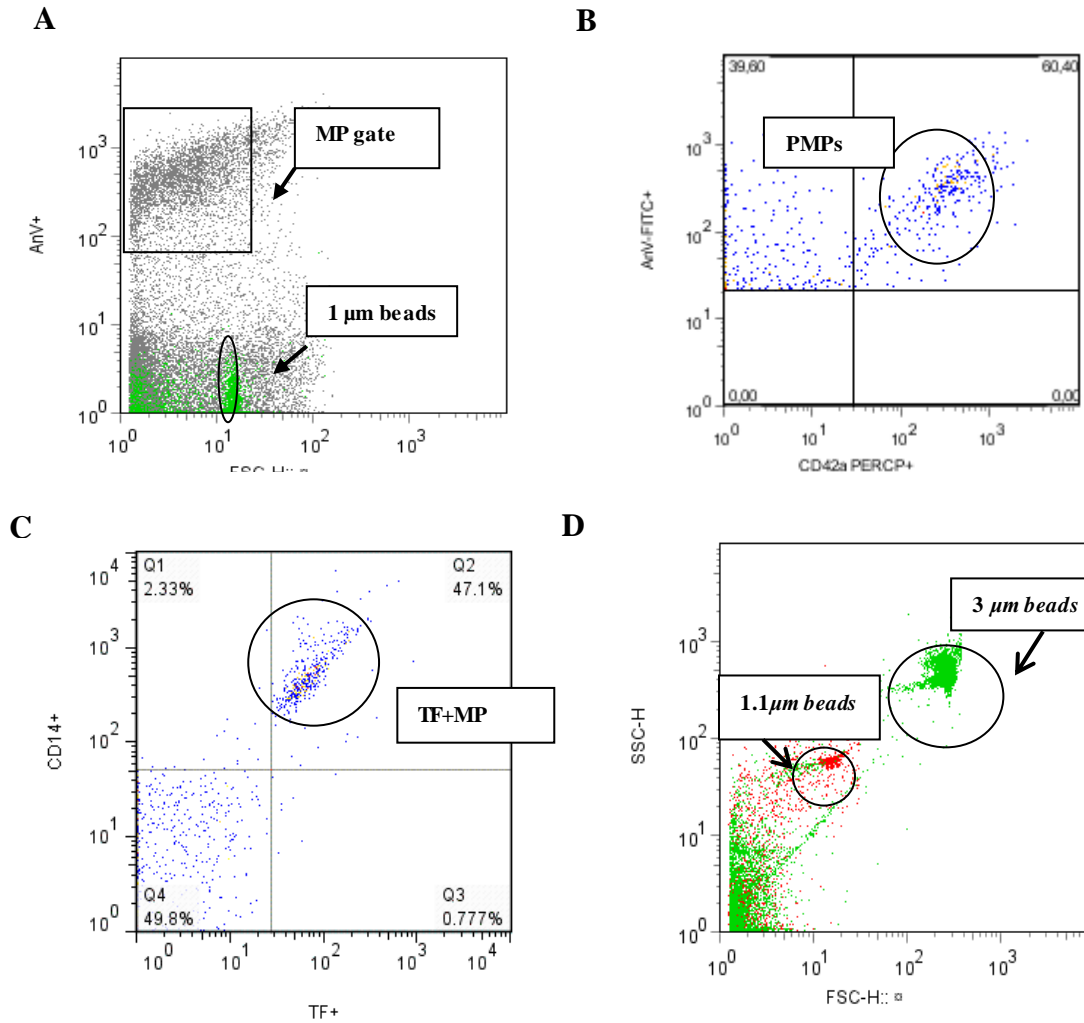


**Figure 4-2: Optimization of gating strategy for microparticle detection.**

(A) Indicates that 0.3, 1.1 and 3  $\mu\text{m}$  beads are distinguishable from the background noise within the system. (B) Signal derived from 0.2  $\mu\text{m}$  pore size filtered water. (C) 0.2  $\mu\text{m}$  beads are not distinguishable based on their FSC and SSC properties but can be detected when labeled with FITC on the FL1 channel. (D) Dot plot demonstrating the gating strategy. The MP gate was defined by forward-scatter characteristics corresponding with a size, 1.1 mm and positive annexin V labelling.

#### ***4.4.5 Flow cytometric analysis of microparticles***

All analysis was performed on a FACScalibur flow cytometer (Becton Dickinson). To obtain optimal forward and side scatter instrument settings for MP 1.1  $\mu\text{m}$  latex beads (Sigma) were run and logarithmic forward and side scatter plots obtained. Gates were then set to include particles less than approximately 1.1 $\mu\text{m}$ , but to exclude the first forward scatter channel containing maximal noise. Optimal compensation was set for green, red and far-red fluorescence. Specific binding for each antibody was determined using isotype control antibodies with equal protein: fluorochrome ratios, and at the same final dilution as per manufacturer recommendation. Since annexin V is a protein and not an antibody (and hence no isotype control antibody exists), the threshold for annexin V binding was determined by using the fluorescence threshold established for MP in the absence labeled annexin V. Particles less than 1.1  $\mu\text{m}$  in size and binding annexin V were then selected (figure 4-3). Platelet derived MPS were defined as particles  $< 1.1 \mu\text{m}$  in size, staining for CD42a+/AnV+ (figure 4-3). Endothelial derived MPs were defined as CD62E+/AnV+/CD42a-, CD54+/AnV+/CD42a-, CD106+/AnV+/CD42a- or CD31+/AnV+/CD42a- MPs. Neutrophil MPs were defined as aCD11b+/AnV+/CD42a- or CD66b+/AnV+/CD42a- MPs and lastly monocyte derived MPs bearing TF or PSGL-1 were identified as TF+/AnV+/CD14+ or PSGL-1+CD14+AnV+ MPs. MP samples were run at medium flow rate with a cut-off time of 1 minute, which resulted in capture of approximately 10000 gated bead events. Beads were used as an internal standard for enumeration- see next section for further details. A representative set of flow cytometric plots, and the gating protocol are shown in figure 4-3. Data were analysed using FlowJo (Treestar Inc, Ashland, OR). The instrument settings are shown in appendix 4.

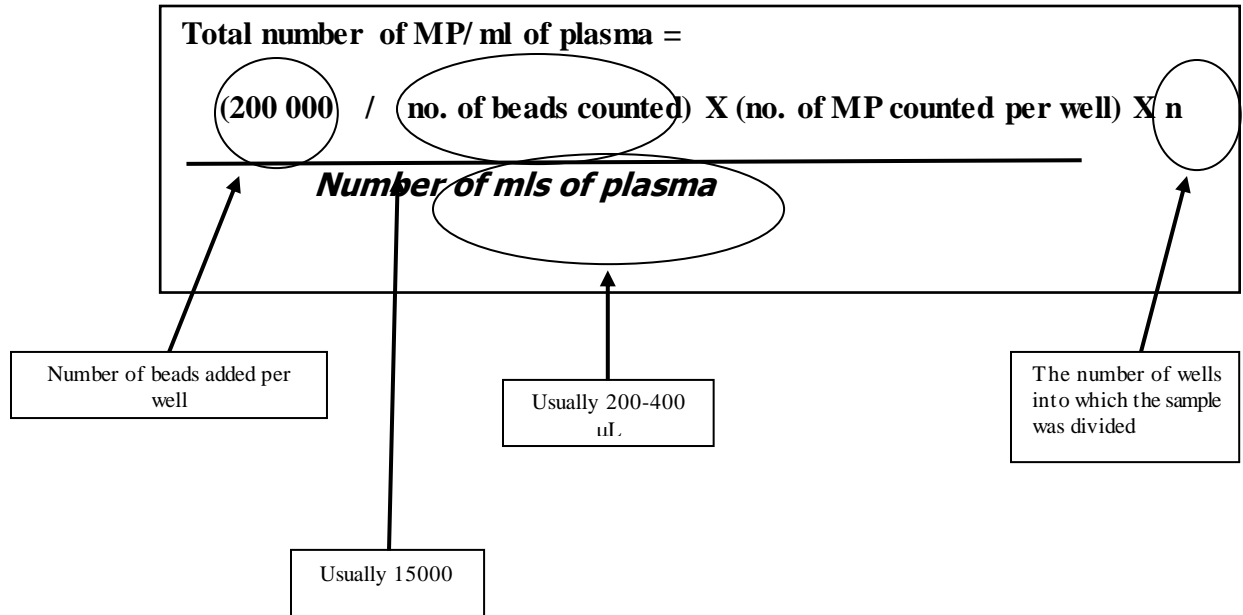


**Figure 4-3: Flow cytometric detection of circulating microparticles (MPs).**

Standard latex beads, size of  $1.1\ \mu\text{m}$  were used to set the gating of  $\text{MP} < 1.1\ \mu\text{m}$ . The MP population with  $\text{AnV}^+$  and size  $< 1.1\ \mu\text{m}$  was selected. Figure 4-3B Platelet MPs (PMPs) were defined as particles  $\text{AnV}^+\text{CD42a}^+$ . Endothelial and neutrophil derived MPs were characterised in an identical way using appropriate monoclonal antibodies. Figure 4-3C Tissue factor positive monocytes derived MPs. Figure 4-3-D  $3\ \mu\text{m}$  beads were run concurrently to enable determination of absolute number of MPs/ml of plasma.

#### ***4.4.6 Determination of absolute microparticle number per ml of plasma***

To convert flow cytometer counts to an estimate of the number of MP per ml of plasma, a predetermined number (always 200000, calculated as per manufacturer recommendations) of 3 µm latex beads (Sigma) were run concurrently with the microparticle samples. The absolute number of annexin V binding microparticles per ml of plasma was then determined by using the proportion of beads counted and the exact volume of plasma from which the microparticles were analysed, as described by Brogan et al. (Brogan et al. 2004). The following equation was thus derived to convert flow cytometer counts to an estimate of the number of MP per ml of plasma (figure 4-4). Since samples from each individual were multiple times, microparticle counts from individual subjects were expressed as the mean number per ml of plasma, with standard error of the mean based on multiple measurements. To determine the absolute number of MP derived from different cellular populations (i.e. platelet, or endothelial) the absolute number of total MP derived from the above equation was multiplied by the percentage positivity for that particular marker.



**Figure 4-4: Conversion equation for calculation of microparticle (MP) number per ml of plasma from flow cytometer counts.**

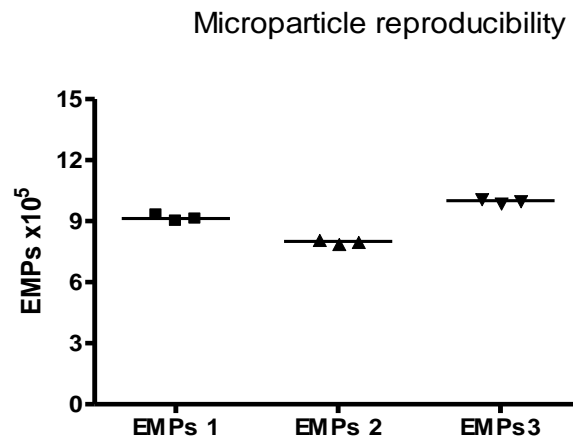
#### **4.4.7 In vitro MP generation as positive controls for set up experiments**

In order to detect the different MP populations in patient samples, MPs were generated in vivo from activated parent cells of varying types. Commercially available Human Umbilical Vein Endothelial Cells (HUVECs), neutrophils, monocytes and platelets isolated from blood donated from adult healthy control volunteers were used for stimulation experiments to induced release of pure endothelial (EMP), neutrophil (NMP), monocyte (MMP) and platelet MPs (PMP). To induce in vitro MP formation, primary cells isolated and cultured as described above in chapter 2 were stimulated with various inflammatory stimuli (for all MP: 10 nM calcium ionophore A23187; EMP: 100ng/ml TNF- $\alpha$  for 24h; NMP: fMLP 10  $\mu$ g/ml for 2 hours; PMP: ADP 5  $\mu$ M for 1hour; MMP: LPS 5 $\mu$ g/ml for 4 hours). MP were isolated from the respective cell cultures after clearance of cell fragments (centrifugation 5000 x g, 5

min) by centrifugation of supernatants (15000 x g for 60 min). The MP pellets were then frozen at  $-80^{\circ}\text{C}$  for future use. The phenotype of MP generated and their activated parent cells is shown in appendix 5.

#### **4.4.8 Reproducibility of MP analysis from different plasma volumes**

To establish the reproducibility of MP analysis, MP derived from supernatants taken from monolayers of HUVEC stimulated with 100 ng/ml of TNF- $\alpha$  (Sigma) at 24 hours from n=3 different experiments were labelled with AnV-FITC and analysed in triplicate. As shown in figure 4-5 each aliquot gave similar mean results for total AnV+ MP enumeration, verifying an acceptable re-reproducibility of the technique of different volumes of frozen plasma.



**Figure 4-5: Microparticle reproducibility.**

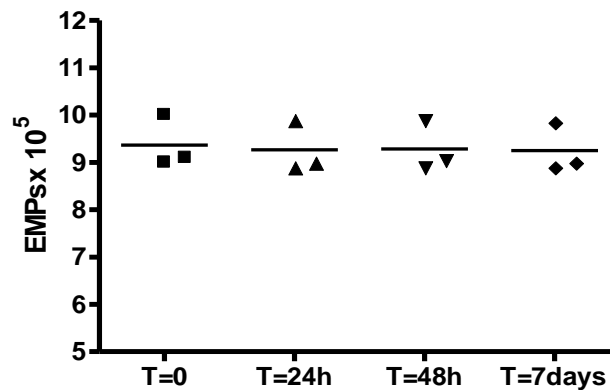
*Supernatants taken from monolayers of human umbilical vein endothelial cells (HUVEC) stimulated with 100 ng/ml of TNF- $\alpha$  for 24 hours from 3 different experiments were labeled with AnV-FITC and analysed in triplicates to provide an estimate of MP reproducibility. EMP=Endothelial derived microparticles.*



#### **4.4.9 Effect of freeze thaw and storage on MP analysis**

The effect of repeat freeze-thaw cycles and storage on the intra-subject reproducibility of total MP number was assessed. MP derived from supernatants taken from monolayers of HUVEC stimulated with 100 ng/ml of TNF- $\alpha$  (Sigma) at 24 hours from n=3 different experiments were analysed at baseline and then frozen and thawed at 24 hours, 48 hours and 7 days of storage. Total AnV+ MP count was not significantly affected by freeze-thaw or storage. Median total AnV+MP count at T=0 was  $9.1 \times 10^5$  (range  $9-10 \times 10^5$  /ml) compared to T=24 h with a median of  $9 \times 10^5$  /ml (range  $8.9-9.9 \times 10^5$  /ml),  $p=0.4$  T=48h median  $9.05 \times 10^5$ /ml (range  $8.9-9.9 \times 10^5$  /ml),  $p=0.7$  and T=7 days median  $9$  (range  $8.9-9.895 \times 10^5$  /ml) $p=0.4$ , figure 4-6.

## Effect of freeze thaw and storage on MP analysis



**Figure 4-6: Effect of freeze thaw and storage on MP analysis.**

Supernatants taken from monolayers of HUVEC stimulated with 100 ng/ml of TNF- $\alpha$  for 24 hours from 3 different experiments were analysed at baseline and then frozen and thawed at 24hours, 48hours and 7 days of storage. MP number was not significantly affected by freeze-thaw. Median MP count at T=0 was  $9.1 \times 10^5$  (range  $9-10 \times 10^5$  /ml) compared to T=7 days median 9 (range  $8.9-9.895 \times 10^5$  /ml),  $p=0.4$ .

## 4.5 Statistical analysis

Numeric results were summarised as median and range. The Kruskal-Wallis test was used to examine overall differences in experimental laboratory markers between the study groups followed by the Mann-Whitney U test. Associations between CEC and MP were assessed using Spearman's rank correlation coefficient. The Wilcoxon matched paired signed ranks test was used to compare MP at initial presentation and at latest follow up for children studied prospectively. The independent association of total annexin V+ MPs on the primary

outcome of AIS recurrence was assessed using a multivariate logistic regression model, unadjusted and adjusted for age, gender, and time from stroke event to blood sampling. Results were expressed as odds ratios (OR) with corresponding 95% confidence intervals (CI) and P-values. P-values of less than 0.05 (two sided) were regarded as significant. Statistical analysis was performed using SPSS versions 16 and 17.

## **4.6 Results**

### ***4.6.1 Circulating cellular microparticles in children with arterial ischaemic stroke***

Circulating MP levels and phenotype in the plasma of children with AIS recurrence or stable disease, cerebral AVM and healthy child controls were compared. Total AnV+ MPs were significantly higher in the 10 children with recurrence at  $659 (136-1616) \times 10^3/\text{ml}$  compared to those with no recurrence  $226 (49-893) \times 10^3/\text{ml}$ ,  $p=0.002$ ; in healthy controls  $89 (25-236) \times 10^3/\text{ml}$ ,  $p=0.0001$  and in children with cerebral AVM  $90 (20-160) \times 10^3/\text{ml}$ ,  $p=0.0001$  (figure 4-7).

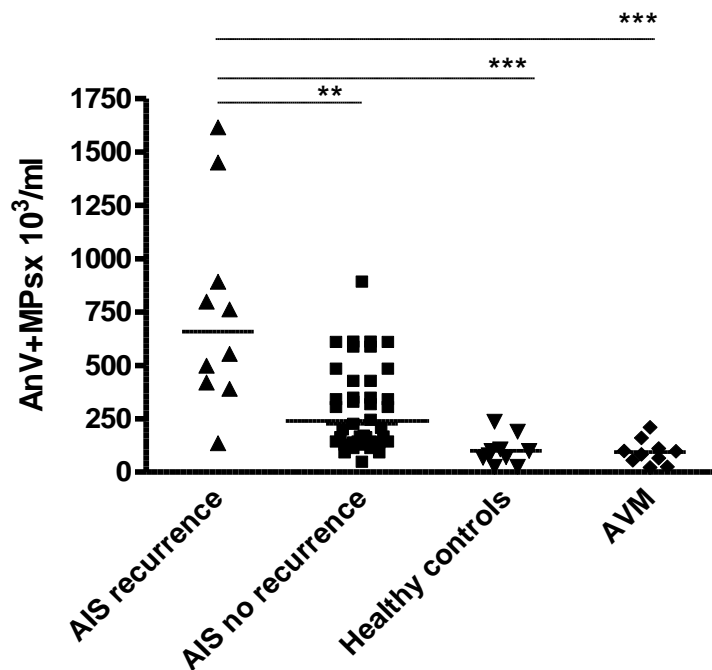
CEC counts correlated significantly with circulating AnV+ MPs,  $r_s=0.45$  ( $p=0.0015$ ; figure 4-8). Platelet derived MP (CD42a+Anv+) were elevated in children with recurrence  $461 (95-980) \times 10^3/\text{ml}$  compared to those with no recurrence  $158 (40-220) \times 10^3/\text{ml}$ ,  $p=0.0010$ ; children with AVM  $80 (45-150) \times 10^3/\text{ml}$ ,  $p=0.0001$  and child controls  $62 (40-120) \times 10^3/\text{ml}$ ,  $p=0.0002$ . Platelet derived MPs expressing CD42a and the platelet activation marker P selectin (CD62P; CD42a+CD62P+ AnV+MPs) were also significantly higher in the group of

children with recurrence:  $93 \times 10^3/\text{ml}$  (18-220  $\times 10^3/\text{ml}$ ), versus the group with no recurrence  $44 \times 10^3/\text{ml}$  (2-120  $\times 10^3/\text{ml}$ ),  $p=0.0100$ ; children with cerebral AVM  $12$  (5-15)  $\times 10^3/\text{ml}$ ,  $p=0.0001$  and child controls  $11 \times 10^3/\text{ml}$  (5-20  $\times 10^3/\text{ml}$ ),  $p=0.0002$  (table 4-2), providing evidence for persistent platelet activation in those with recurrence. In addition, endothelial MPs (EMPs) expressing the endothelial activation marker CD62E (E-selectin) but negative for the platelet marker CD42a (CD62E+CD42a-AnV+ EMPs) were significantly higher in children with recurrence  $85 \times 10^3/\text{ml}$  (44-164  $\times 10^3/\text{ml}$ ) compared to children with no recurrence  $38 \times 10^3/\text{ml}$  (1-105  $\times 10^3/\text{ml}$ ),  $p=0.0001$ ; children with cerebral AVM  $15 \times 10^3/\text{ml}$  (2-35)  $\times 10^3/\text{ml}$ ,  $p=0.002$ ; and healthy controls  $0.5 \times 10^3/\text{ml}$  (0.2-10  $\times 10^3/\text{ml}$ ),  $p=0.0001$ , (table 4-2). Similar significant differences were seen for EMPs expressing CD31+ between the two patient groups,  $80 \times 10^3/\text{ml}$  (55-130)  $\times 10^3/\text{ml}$  for those with recurrence compared to  $40 \times 10^3/\text{ml}$  (15-90)  $\times 10^3/\text{ml}$ ,  $p=0.002$  for those with a single event; for children with cerebral AVM  $5 \times 10^3/\text{ml}$  (2-10)  $\times 10^3/\text{ml}$ ,  $p=0.003$  and  $2 \times 10^3/\text{ml}$  (1-9)  $\times 10^3/\text{ml}$  for child controls,  $p=0.0001$ . There were no significant differences between groups for EMP expressing CD 106,  $p=0.548$ . Of note circulating EMPs expressing CD62E correlated significantly with CECs,  $r_s=0.67$ ,  $p=0.0001$  suggesting a significant association between both biomarkers of endothelial injury (figure 4-9).

Neutrophil derived MPs defined as aCD11b+CD42a-AnV+ MPs were significantly higher in the group with recurrence  $60 \times 10^3/\text{ml}$  (8-125  $\times 10^3/\text{ml}$ ) compared to the group with no recurrence  $21 \times 10^3/\text{ml}$  (1-40  $\times 10^3/\text{ml}$ ),  $p=0.0200$ ; children with cerebral AVM  $19 \times 10^3/\text{ml}$  (1-40)  $\times 10^3/\text{ml}$ ,  $p=0.0030$ ; and controls  $4 \times 10^3/\text{ml}$  (0.5-17  $\times 10^3/\text{ml}$ ),  $p=0.0040$  providing evidence for neutrophil activation (table 4-2).

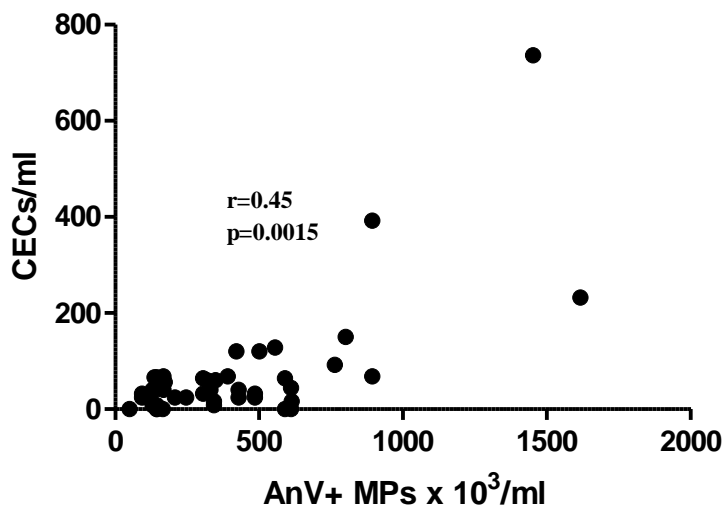
Tissue factor (TF) expression on MP of monocytic (CD14) origin was then compared between the groups since these are known to be highly prothrombotic. TF+CD14+AnV+ MPs were significantly elevated in those with AIS recurrence  $70 (22- 190) \times 10^3/\text{ml}$  compared to these with a single event  $29 (1-146) \times 10^3/\text{ml}$ ,  $p=0.0040$ ; healthy control children  $2 (0.2- 6.7) \times 10^3/\text{ml}$ ,  $p=0.0001$ ; and children with cerebral AVM  $10 (2-30) \times 10^3/\text{ml}$ ,  $p=0.0030$ , table 4-2.

As TF activity at the thrombus edge has been previously shown to depend on interactions between platelet P-selectin and P-selectin glycoprotein ligand -1 (PSGL-1)(Falati et al. 2003) on monocyte MPs I then determined whether there were any differences in PSGL-1+CD14+AnV+ MPs between the patient groups. The AIS recurrence group had significantly higher PSGL-1+CD14+AnV+ MPs of  $35 \times 10^3/\text{ml}$  ( $15-98 \times 10^3/\text{ml}$ ) compared to  $12 \times 10^3/\text{ml}$  ( $5 -58 \times 10^3/\text{ml}$ ),  $p=0.0200$  in the group of those children with no recurrence; in the children with cerebral AVM  $5 \times 10^3/\text{ml}$  ( $1.2-8.2$ ) $\times 10^3/\text{ml}$ ,  $p=0.003$  and  $1.3 \times 10^3/\text{ml}$  ( $1.1-1.5 \times 10^3/\text{ml}$ ),  $p=0.0200$  in healthy controls.



**Figure 4-7: Circulating total Annexin V+microparticles in children with arterial ischemic stroke (AIS).**

Total Annexin V+ (phosphatidylserine rich) microparticles were significantly elevated in children with AIS compared to those with a single event ( $p=0.002$ ) and children with cerebral arteriovenous malformation (AVM;  $p=0.0001$ ) and healthy child controls ( $p=0.0001$ ).  $***P<0.0005$  with Mann Whitney U test.

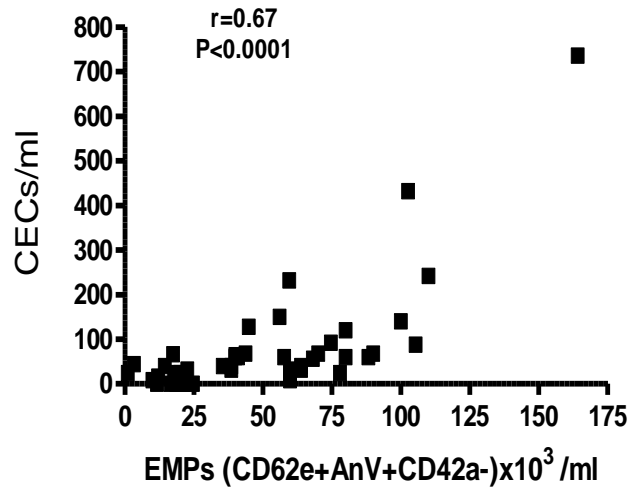


**Figure 4-8: Circulating endothelial cell counts (CEC) and total Annexin V+ microparticles (MP).** CEC correlated significantly with circulating Annexin V+ MP ( $r_s=0.45$ ;  $p=0.0015$ ).

<b>Microparticles (median, range) x 10<sup>3</sup>/ml</b>	<b>AIS recurrence N=10</b>	<b>AIS no recurrence N=36</b>	<b>Healthy controls N=10</b>	<b>Cerebral AVM N= 10</b>
Total AnV+MPs	659 (136-1616)	226 (49-893); <b>p=0.002</b>	89 (25-236); <b>p=0.0001</b>	90 (20-160); <b>p=0.0001</b>
EMP AnV+CD62E+CD42a-	85 (44-164)	38 (1-105); <b>p=0.0001</b>	0.5 (0.2- 10); <b>p=0.0001</b>	15(2-35); <b>p=0.002</b>
EMP AnV+CD31+CD42a-	80 (55-130)	40 (15-90); <b>p=0.002</b>	2 (1-9); <b>p=0.0001</b>	5 (2-10); <b>p=0.003</b>
PMP AnV+CD42a+	461(95-980)	158 (40-220); <b>p=0.0010</b>	62 (40-120); <b>p= 0.0002</b>	80(45-150); <b>p=0.0001</b>
PMP AnV+CD42a+CD62P+	93 (18 -220)	44 (2-120); <b>p=0.0100</b>	11 (5-20); <b>p= 0.0002</b>	12 (5-15); <b>p=0.0001</b>
NMP AnV+aCD11b+CD42a-	60 (8-125)	21 (1-40); <b>p=0.0200</b>	4 (0.5-17); <b>p=0.0040</b>	19 (1-40); <b>p=0.0030</b>
TF+CD14+AnV+	70 (22- 190)	29 (1-146); <b>p=0.0040</b>	2 (0.2- 6.7); <b>p=0.0001</b>	10 (2-30); <b>p=0.0030</b>
PSGL-1+ CD14+AnV+	35 (15-98 )	12 (5-58); <b>p=0.0200</b>	1.3 (1.1-1.5); <b>p=0.0200</b>	5 (1.2-8.2); <b>p=0.0030</b>

**Table 4-2: Circulating microparticle profiles in children with arterial ischaemic stroke, cerebral arteriovenous malformation and child healthy controls.**

*AIS=arterial ischaemic stroke; AVM=arteriovenous malformation; EMP=endothelial derived MP; PMP=platelet derived MP; NMP=neutrophil derived MP; TF=tissue factor; PSGL-1=P selectin glycoprotein ligand-1. The Mann Whitney U test was used to compare MP counts between groups. P values refer to comparison with the group of children with recurrent AIS.*

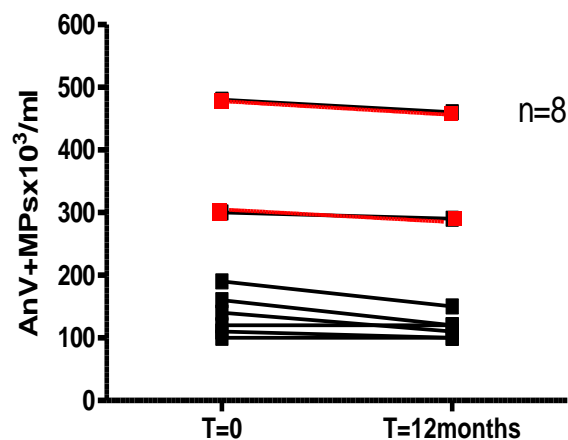


*Figure 4-9: Circulating endothelial cell counts (CEC) and endothelial microparticles (EMP). Circulating EMPs expressing the activation marker CD62E correlated significantly with CEC,  $r_s=0.67$ ,  $p=0.0001$  suggesting a significant association between both biomarkers of endothelial injury.*



#### **4.6.2 Longitudinal changes of microparticles (MP) in children with arterial ischaemic stroke**

Figure 4-10 summarises the longitudinal changes in total AnV+ MPs in 6 children with a stable course with no AIS recurrence:  $130 \times 10^3/\text{ml}$ , range  $120\text{-}190 \times 10^3/\text{ml}$  at initial assessment to  $115 \times 10^3/\text{ml}$ , range  $120\text{-}150 \times 10^3/\text{ml}$  at follow up and in 2 children with recurrence: median 390, range  $300\text{-}480 \times 10^3/\text{ml}$  at presentation to a median of 375, range  $290\text{-}440 \times 10^3/\text{ml}$  at follow up ( $p=0.3$ ). Of note the 2 children with recurrence had been treated with aspirin and warfarin but no immunosuppression.



**Figure 4-10: Longitudinal changes in total Annexin V+ microparticles (MP) in children with arterial ischaemic stroke (AIS) and cerebral arteriopathy.**

Total AnV+ microparticles in 6 children with a stable course with no AIS recurrence and in 2 children with recurrence. The 2 children with recurrence had been treated with aspirin and warfarin but no immunosuppression.

### **4.6.3 Logistic regression analysis of annexin V microparticles for arterial ischaemic stroke recurrence**

Logistic regression analysis was used to examine the relationship of total AnV+MPs to AIS recurrence. Table 4-3 gives the unadjusted odds ratios, and odds ratios adjusted for age, gender, and time from recent AIS event to blood sampling. Total AnV+MPs were significantly associated with AIS recurrence in the unadjusted model (OR 1.410 95% CI 1.100-1.545, p=0.004) and remained significant in the adjusted analysis, (OR 1.220; 95% CI 1.039-1.317, p= 0.020).

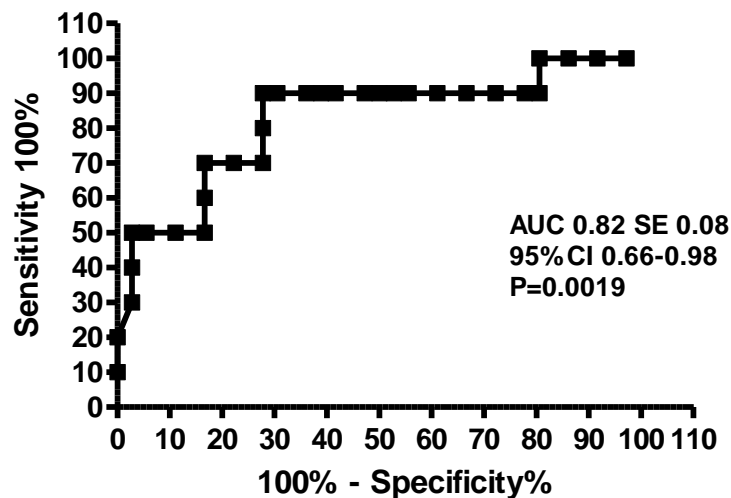
<b>Biomarker</b>	<b>Unadjusted</b>			<b>Adjusted (age/gender/time from AIS to evaluation)</b>		
	<b>OR</b>	<b>95%CI</b>	<b>P</b>	<b>OR</b>	<b>95%CI</b>	<b>P</b>
Annexin V+ microparticles (AnV+MPs)	1.410	1.100-1.545	0.004	1.220	1.039-1.317	0.020

**Table 4-3: Unadjusted and adjusted odds ratios by multivariable logistic regression analysis of total Annexin V+ microparticles (MP) for arterial ischaemic stroke recurrence (AIS).**

*Adjusted odd ratios for age/gender/time from AIS to evaluation. OR=odds ratio; CI=confidence intervals. P<0.05 was considered significant.*

#### **4.6.4 Test characteristics of MPs for identification of AIS recurrence**

On the basis of this regression analysis demonstrating that total AnV+ MPs strongly associated with recurrent AIS, the test characteristics of MPs for identification of AIS recurrence were then examined by plotting ROC curves. The ROC curve for MPs at varying definitions of positivity is shown in figure 4-11. ROC analysis for MPs as a diagnostic test for identification of recurrence was significant with an AUC of 0.8236, SE 0.08 and 95% CI of 0.66-0.98,  $p=0.0019$ . The full test characteristics of MPs for identification of AIS recurrence are summarized in table 4-4. The cut-off values for test positivity in table 4-4 correspond to individual points on the ROC curves.



*Figure 4-11: Receiver operator characteristic curve (ROC) for total Annexin V positive (PS rich) microparticles for the identification of arterial ischaemic stroke (AIS) recurrence. ROC analysis was significant with an area under the curve (AUC) of 0.82 (95% CI 0.66-0.98),  $p=0.0019$ .*

<b>AnV+ MPs</b>	<b>Sensitivity (%)</b>	<b>95% CI</b>	<b>Specificity (%)</b>	<b>95% CI</b>	<b>LR+</b>	<b>LR-</b>	<b>PPV</b>	<b>NPV</b>
> 70.93	100	69- 100	2.778	0-14	1.03	0	50	100
> 102.7	100	69-100	8.333	2-22	1.09	0	52	100
> 121.2	100	69- 100	13.89	5-29	1.16	0	53	100
> 133.1	100	69-100	19.44	8-36	1.24	0	55	100
> 138.3	90	55-99	19.44	8-36	1.12	0.51	52	65
> 141.9	90	55-99	22.22	10-39	1.16	0.45	53	68
> 143.9	90	55-99	27.78	14- 45	1.25	0.35	55	73
> 154.3	90	55-99	33.33	18-51	1.35	0.30	57	76
> 165.8	90	55-99	38.89	23-56	1.47	0.25	59	79
> 169.2	90	55-99	44.44	27- 61	1.62	0.22	61	81
> 189.2	90	55-99	47.22	30-64	1.71	0.21	63	82
> 226.7	90	55-99	50	32-67	1.8	0.20	64	83
> 275.7	90	55-99	52.78	35-69	1.91	0.19	65	83
> 311.7	90	55-99	58.33	40-74	2.16	0.17	68	85
> 324.5	90	55-99	61.11	43-76	2.31	0.16	69	85
> 336.5	90	55-99	63.89	46-79	2.49	0.15	71	86
> 345.6	90	55-99	69.44	51- 83	2.95	0.14	74	87
> 369.9	90	55-99	72.22	54-85	3.24	0.13	76	87
> 406.0	80	44-97	72.22	54-85	2.88	0.27	74	74
> 424.5	70	34- 93	72.22	54-85	2.52	0.41	71	70
> 456.5	70	34-93	77.78	60-89	3.15	0.38	75	71
> 492.5	70	34- 93	83.33	67-93	4.2	0.36	80	73
> 527.7	60	26-87	83.33	67- 93	3.6	0.48	77	67
> 572.2	50	18-81	83.33	67-93	3	0.60	74	62
> 600.0	50	18-81	88.89	73-96	4.5	0.56	96	63
> 611.7	50	18-81	94.44	81-99	9	0.52	89	65
> 687.6	50	18-81	97.22	85-99	18	0.51	94	65

> 781.4	40	12-73	97.22	85-99	14.4	0.61	94	70
> 846.6	30	6-65	97.22	85-99	10.8	0.72	90	58
> 1173	20	2-55	100	90-100	Infin ity	0.80	100	55
> 1534	10	0-44	100	90-100	Infin ity	0.90	100	52

***Table 4-4 Test characteristics of total Annexin V positive microparticles for identification of arterial ischaemic stroke (AIS) recurrence.***

*PPV= positive predictive value; NPV= negative predictive value; LR+ = Likelihood ratio for a positive test result; LR- = Likelihood ratio for a negative test result.*

## **4.7 Discussion**

Total circulating AnV+ (PS-rich) MPs were higher in children with AIS recurrence than in those with a stable disease course (figure 4-7). These included increased AnV+MPs of activated platelet (expressing CD62P), endothelial (expressing CD62E, with strong correlation to CECs) and neutrophil (CD11b) origin. Notably, monocytic MPs expressing tissue factor and PSGL-1 were significantly raised in the circulation of children with AIS recurrence conferring an increased pro-thrombotic risk in these children. These novel findings suggest that circulating MP profiles effectively differentiate between subtypes of childhood AIS and provide useful insights into endothelial, platelet, monocyte and neutrophil activation as major contributors to the pathogenesis of AIS recurrence.

EMP are released from endothelial plasma membrane blebbing and carry a variety of endothelial proteins such as intercellular cell adhesion molecule (ICAM)-1, E-selectin, endoglin, vascular endothelial cadherin, platelet endothelial cell adhesion molecule-1 (PECAM-1)(Dignat-George & Boulanger 2011). Determining the endothelial origin of circulating MP involves the use of specific markers for flow cytometry analysis. Of note however some of these surface markers such as PECAM-1 (CD31) are not exclusively expressed on endothelium (Newman 1997). Hence the need to develop strategies combining multiple markers to exclude possible contaminating subpopulations and accurately assess the endothelial origin of MP in biological fluids. In this study additional staining with CD42-a (a constitutively expressed platelet marker) was performed for this reason. Although EMP represent a sparse population of circulating MP, changes in their plasma levels reflect an active balance between MP generation and clearance and might carry important clinical information in healthy subjects and in patients with cardiovascular disorders (Dignat-George & Boulanger 2011; Koga et al. 2005; VanWijk et al. 2003; Werner et al. 2006). So far several studies have investigated the prognostic potential of the measurement of EMP plasma levels (Dignat-George & Boulanger 2011; Koga et al. 2005; VanWijk et al. 2003; Werner et al. 2006). In patients with coronary artery disease levels of circulating EMP relate to endothelial dysfunction independently of age and blood pressure (Koga et al. 2005; Werner et al. 2006). In adult patients with acute ischaemic stroke, EMP levels are associated with lesion volume and clinical outcome (Jung et al. 2009; Simak et al. 2006). In pulmonary hypertension, circulating EMP expressing E-selectin predicted the 1-year outcome of these patients (Bakouboula et al. 2008). These data suggest that EMP levels are a biomarker of

endothelial injury and may be used for stratification of patients with a high risk of developing cardiovascular complications.

Similarly, in this cohort of children with AIS, EMPs expressing a variety of endothelial surface markers were significantly elevated in those with recurrence and arteriopathy progression compared to children with a single event. Importantly, children do not have atherosclerosis, which may be a confounding factor when studying endothelial microparticles in adult disease states. In addition EMP correlated well with CEC suggesting that both these indices permit the study of the endothelium in vivo although a direct causal link between these parameters remains to be confirmed and these are unlikely to be independent biomarkers since both relate to endothelial injury and/or activation.

Due to their high membrane plasticity and high membrane surface area platelets are the main provider of circulating MPs in healthy individuals (Morel et al. 2006; Morel et al. 2008b). In other cells scrambling is not as effective, with the lowest ability for MP released being found in red blood cells (Morel et al. 2006; Piccin, Murphy, & Smith 2007). An increased number of microparticles of platelet origin expressing CD42a in the group of children with AIS recurrence were observed in this study comprising 70-80 % of the whole MP population. The PMP were previously reported to be increased in both acute and chronic phase of cerebral infarction in adults. Cherian et al. demonstrated a correlation between the elevated blood PMP and endothelial dysfunction markers like P-selectin and E-selectin in acute phase of cerebral infarction (Cherian et al. 2003). Kuriyama et al. additionally showed that elevated PMP level significantly correlated with intima media thickness and with

concomitant intracranial stenosis of carotid arteries (Kuriyama et al. 2010). The levels of PMP were also significantly elevated in the patients with cerebral infarction in anterior, posterior, middle cerebral arteries, and lacunar stroke in contrast with the group of the patients with cardioembolic stroke (Kuriyama et al. 2010). Of note, in this study there were also significant differences between patient groups in PMP expressing the activation marker P-selectin, known to play an important role in the recruitment and aggregation of platelets through platelet-fibrin and platelet-platelet binding at areas of vascular injury (Kuriyama et al. 2010). Additionally other mechanisms triggered by P-selectin could contribute to increased thrombotic propensity. P-selectin facilitates the transfer of TF sorted from raft into monocyte-derived MPs and this is then delivered as a functional entity to platelets (Del Conde, Shrimpton, Thiagarajan, & López 2005b). P-selectin also promotes PS exposure by monocytes and TF expression (Del Conde et al. 2005a). Finally, Mesri et al. have shown that P-selectin stimulation of monocytes generated MP enriched in lymphocyte function-associated antigen-1 (LFA-1; CD11a/CD18) that enable interactions between leukocytes and endothelial cells (Mesri & Altieri 1998; Mesri & Altieri 1999). Of note, high plasma levels of soluble P-selectin were previously demonstrated to be predictive of venous thromboembolism in several diseases (Ay et al. 2008; Kyrle et al. 2007). Thus the release and levels of circulating PMP relate to platelet activation and are likely to have direct functional significance in ischaemic vascular diseases such as childhood AIS.

Additionally, elevated numbers of NMP expressing the activation epitope aCD11b were observed in children with AIS recurrence. Neutrophil microparticles have not been previously studied in AIS or other cerebrovascular diseases. Most experimental and clinical



studies however support the importance of neutrophil infiltration in ischaemic stroke (Bednar et al. 1991; Emerich et al. 2002; Kochanek and Hallenbeck 1992). The brain's inflammatory response to ischaemia is well characterized by a rapid activation of resident cells (mainly microglial cells), followed by the infiltration of circulating inflammatory cells, including neutrophils, T cells, monocyte/ macrophages as demonstrated in animal model (Amantea et al. 2009; Jin et al. 2010; Schilling et al. 2003 Tanaka et al. 2003). Genetic deficiency or antibody blockade of leukocyte adhesion molecules (e.g., ICAM-1, CD11b/CD18, P-selectin) has been shown to reduce infarct volume, brain oedema, neurological deficits, and mortality in animal models of ischaemic stroke (Jin, Yang, & Li 2010; Kitagawa et al. 1998; Zhang et al. 2003; Zhang et al. 1995). Furthermore, studies in humans have suggested that neutrophil accumulation is correlated with the severity of the brain tissue damage and poor neurological outcome after ischaemic stroke (Akopov et al. 1996; Jin, Yang, & Li 2010; Price et al. 2004). Potential mechanisms that may explain how activation and accumulation of neutrophils contribute to the pathogenesis of ischaemic stroke include: excessive production of ROS, such as superoxide and hypochlorous acid via NADPH oxidase and MPO, respectively; and release of a variety of proinflammatory cytokines and chemokines and enhancing expression of adhesion molecules (PSGL-1, L-selectin) (Jin, Yang, & Li 2010). By these mechanisms, infiltrating neutrophils further amplify cerebral inflammatory responses. NMP have also been previously shown to exert potent inflammatory responses into endothelial cells, induce oxidative stress and impair angiogenesis (Hong et al. 2012). Of note neutrophil infiltration may play a more prominent role in the pathogenesis of ischaemic stroke in individuals with elevated systemic inflammation such as in patients with prior infection (Amlie-Lefond et al. 2009; Fullerton et

al. 2011). This is of considerable interest for the pathogenesis of childhood AIS particularly in view of the fact that several studies have previously suggested a link between common systemic infections and the development of cerebral arteriopathies leading to AIS in children. In this regard, it is critical to better understand the exact roles of circulating neutrophils and perhaps NMP as a window to neutrophil activation in the pathogenesis of ischaemic stroke under clinically relevant conditions.

Intriguingly, elevated levels of monocytic MP expressing TF and PSGL-1 were observed in children with AIS recurrence and may contribute to the thromboembolic events associated with AIS. PS on the outer surface of MPs directly contributing to activation of the extrinsic coagulation pathway by activating factor X but also indirectly by enhancing the capability of tissue factor (TF)(Bach 2006; Giesen et al. 1999). Further pro-thrombotic potential of circulating MPs could be mediated via interaction of TF+PSGL1+ monocyte MPs and P selectin+ PMPs, as previously suggested by animal data supporting the delivery of TF in the thrombus edge via the aforementioned MPs (Falati et al. 2003). Falati et al. using intravital real time microscopy have established that the swift recruitment at the edge of the thrombus of leukocytes and leukocyte-derived MPs through P-selection/P-SGL-1 interactions promotes thrombus growth (Falati, Gross, Merrill-Skoloff, Furie, & Furie 2002). The importance of such interactions was confirmed in mice lacking PSGL-1 or P-selectin, TF accumulation within the thrombus being minimal (Falati, Gross, Merrill-Skoloff, Furie, & Furie 2002). We confirmed the presence of TF+ and PGSL-1+ monocyte MPs and P selectin+ PMPs in higher numbers in the plasma of children with arteriopathy progression. These observations apart from providing diagnostic information offer additionally an

intriguing pathophysiological insight into the hitherto undefined thrombotic propensity underpinning childhood AIS recurrence and will be considered in more detail in chapter 5.

On the whole, the test characteristics of circulating microparticles for prediction of AIS recurrence based on a limited number of children with AIS are encouraging. A number of endothelial surface markers of varying specificity were utilised to characterise the microparticles as being of endothelial or platelet origin. Furthermore longitudinal measurements may be useful in individual patients to monitor disease activity and predict recurrence. The preliminary prospective data presented here suggest that MP in children at risk of recurrence remain elevated and may contribute to thrombosis. Examining the changes of MP at several time points during the disease course for individual patients may provide additional information on the activatory phase of the disease and effect of treatment. Future studies may also give insights into the origin of EMP in AIS, to establish whether these MPs are from generalized endothelial stimulation or from local ischaemia and stimulation, or both. Overall however the study of EMP permits the status of the endothelium to be studied in vivo, providing a novel approach that has promising potential for further understanding of stroke pathophysiology

The use of flow cytometry to characterise MP subpopulations undoubtedly has some limitations. MP at the lowest range of size are likely to have been beyond the detection limits of flow cytometry, particularly since standard protocols set thresholds to exclude the first FSC channel to limit background noise. In addition, a multiparametric approach with combination of other surface markers would provide additional information regarding MP

phenotype, particularly in relation to activation status and more definitive data regarding MP subpopulations. Latex beads were used for an approximation of size but circulating MP may have different optical properties hence the gating strategy employed herein may need further optimisation (Yuana, Bertina, & Osanto 2011). New approaches such as impedance based flow cytometry where the labelling efficiency is independent of the particle shape and (surface) composition may help overcome these difficulties but are yet in their infancy (Yuana, Bertina, & Osanto 2011). Of note, MPs externalize PS and thus bind annexin V in a calcium-dependent manner. However, annexin V-negative membrane vesicles expressing endothelial or other cell markers have been identified in human plasma, suggesting that circulating MP may not all externalize PS, or that for yet unknown reasons, PS is unavailable for annexin V binding (Perez-Pujol et al. 2007; Yuana, Bertina, & Osanto 2011). Staining with lactadherin, a milk-derived protein maybe more sensitive to small changes in PS expression than annexin V and it can detect PS-positive MPs in a calcium-free environment, such as citrate- or EDTA-anticoagulated plasma samples (Hou et al. 2011; Lacroix, Robert, Poncelet, & Dignat-George 2010a). Of interest, these MPs with reduced PS exposure may be evading macrophage removal from the peripheral circulation and could be important pathogenic mediators that merit further study.

In conclusion significant differences were observed in circulating endothelial, platelet, monocyte and neutrophil MP signatures between patient groups, and differentiate patients with stroke recurrence from those with a single event. These observations complement the findings relating to CEC described in previous sections. These findings not only provide additional evidence for ongoing endothelial injury and platelet activation in those with

recurrent AIS, but could also have major pathophysiological implications. The functional relevance of circulating MP to the pathogenesis of AIS particularly with respects to their procoagulant activity contributing to the thromboembolic phenomena associated with re-infarction is examined in more detail in chapter 5.

# **5 Microparticle-mediated thrombin generation: novel means of assessing microparticle related procoagulant activity**

## **5.1 Summary**

**Background:** Microparticles (MPs) are membrane fragments rich in phosphatidylserine and have been previously shown to be elevated in a wide range of vascular disorders including systemic vasculitis and now as shown in chapter 4 childhood AIS. However the functional significance of these findings particularly the relative contribution of MP to the thrombotic propensity associated with these conditions remains to be defined. As thromboembolic disease is a well established complication of systemic vasculitis the prothrombotic potential of MP in this systemic vascular inflammatory disorder is examined first prior to using a similar approach in childhood arteriopathic AIS a condition confined to the cerebral vasculature.

**Objective:** Assess the pro-coagulant activity of plasma MPs in children with active systemic vasculitis and in relation to thromboses.

**Methods:** Children with systemic vasculitis with or without thromboses were studied during active or inactive disease as assessed by the Birmingham Vasculitis Activity Score (BVAS) and evidence of endothelial injury (circulating endothelial cells, CECs). Annexin V+ MPs were quantified using flow cytometry. The MP related thrombin generating capacity was quantified using a novel fluorometric thrombin generation assay.

**Results:** 25 children of median age 8 (1.2-16.1) years with vasculitis were studied. Fifteen children with active disease (BVAS 7/63, range 5-20/63; CECs 240/ml, range 112-1600 cells/ml) exhibited significantly higher peak thrombin of 145.85 nM, (80.4-189.2 nM) compared to 10 children with inactive vasculitis (BVAS=0/63 in all; CECs 46/ml, range 40-112 cells/ml): peak thrombin 53.6 nM (45-131.4 nM),  $p=0.002$ ; and healthy age-matched controls: peak thrombin 45 nM (24-60 nM),  $p=0.001$ . Peak thrombin correlated significantly with the total number of plasma MPs,  $r_s=0.83$ ,  $p=0.0001$ . Children ( $n=7$ ) with vasculitis and thrombosis had higher peak thrombin than those without,  $p=0.0184$ . Receiver operator characteristic analysis of peak thrombin as a tool to diagnose thrombosis was significant,  $p<0.0005$ .

**Conclusions:** Enhanced MP-mediated thrombin generation was demonstrated in children with active vasculitis, and was even higher in those with thrombosis. MP-mediated thrombin generation combined with endothelial injury could account for the thrombo-embolic complications in vasculitis. MP mediated thrombin generation assay provides novel means of assessing thrombotic propensity.

## **5.2 Introduction**

### ***5.2.1 Thrombosis in systemic vasculitis***

Thromboembolic disease is a serious complication of systemic vasculitis and is associated with significant morbidity and mortality (Merkel et al. 2005; Tomasson et al. 2009; Weidner et al. 2006). In particular arterial and venous thrombosis leading to ischaemic and/or

embolic events complicate Kawasaki disease (KD), polyarteritis nodosa (PAN), Takayasu arteritis (TA), Behçet's disease (BD), and more recently have been reported in small vessel vasculitides such as antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) (Houman et al. 2001; Kato et al. 1986; Tomasson, Monach, & Merkel 2009). Merkel et al. in 2005 reported on the incidence of venous thrombotic events (VTE) in patients with AAV enrolled in the Wegener's Granulomatosis Etanercept Trial (WGET) in the United States. All VTEs were clinically apparent and confirmed with diagnostic studies (Merkel et al. 2005). Among the 167 patients without a history of VTE, 16 experienced events during 228 person-years of observation, an incidence of 7.0 VTEs per 100 person-years (95% CI, 4.0 to 11.4) (Merkel et al. 2005). These thrombotic events included either deep venous thrombosis (DVT) of lower extremities or pulmonary embolism (Merkel et al. 2005). Notably, most VTEs occurred during or closely following periods of active disease (Merkel et al. 2005). In addition, Weidner et al. retrospectively reviewed all patients who were treated for AAV at a single nephrology clinic during a 16-year period (Weidner et al. 2006). Thirteen of 105 patients had VTEs during the 367.5 person-years of observation, resulting in an incidence of 4.3 events per 100 person-years (Weidner et al. 2006). Importantly, no patients with VTEs in this cohort were found to have protein C, protein S, or antithrombin III deficiencies, antiphospholipid antibodies, factor V Leiden mutation, or nephrotic syndrome (Weidner et al. 2006). In another study, Stassen et al. looked at VTE in a cohort of 198 patients with AAV (Stassen et al. 2008). During a median follow-up period of 6.1 years, 25 VTEs occurred in 25 patients, an incidence of 1.8 events per 100 person years (Stassen et al. 2008). Similar to what was found in the two above-outlined studies, a much



higher incidence of thrombosis (6.7 per 100 person years) was observed during periods of active disease (Asahara et al. 1999b; Rehman et al. 2003).

Despite this well established thrombotic propensity associated with vasculitis, the underlying mechanism of hypercoagulability remains however poorly defined. For instance the prevalences of antiphospholipid antibodies and common genetic mutations associated with hypercoagulability were recently determined among individuals in the WGET study (Sebastian et al. 2007). Although there was a slightly increase prevalence of antiphospholipid antibodies among persons with granulomatosis with polyangiitis (GPA; formerly known as Wegener's granulomatosis) compared with the general population (confirming prior reports), there was no association found between the presence of anticardiolipin antibodies and VTE (Sebastian et al. 2007). Additionally, the same study showed that the prevalences of mutations in factor V Leiden, prothrombin G20210A, and methylenetetrahydrofolate reductase were not different among patients with GPA and VTE compared with rates in the general population (Sebastian et al. 2007). Therefore these reports have not demonstrated any increased prevalence of traditional acquired or genetic thrombophilia in patients with vasculitis complicated by thromboses compared to the general population.

Vasculitis could increase thrombotic risk via several mechanisms (Libby and Simon 2001; Trifiletti et al. 2009). The initiating event in activation of the coagulation cascade *in vivo* is expression of active tissue factor (TF), which leads to serial activation of individual coagulation factors that lead to fibrin generation and formation of a thrombus (figure 5-1)

(Furie and Furie 2008; Nemerson 1988). Active TF comes in contact with the other components of the coagulation cascade through two main pathways. First, disruption of the endothelial layer as a result of vascular injury exposes active TF on the underlying connective tissue (Rauch and Nemerson 2000; Tomasson, Monach, & Merkel 2009). Secondly several inflammatory cytokines that can induce TF expression on endothelial cells and circulating monocytes are secreted in systemic vasculitis that may upregulate TF expression on endothelial cells and monocytes (Rauch & Nemerson 2000; Tomasson, Monach, & Merkel 2009). In addition, activated platelets could also play role in thrombosis in vasculitis. Activated platelets release several cytokines including CD40 ligand and vascular endothelial growth factor (VEGF), which then stimulate coagulation by induction of TF expression on both monocytes and endothelial cells (Henn et al. 1998; Slupsky et al. 1998; Tomasson, Monach, & Merkel 2009). Other potential mechanisms of association between systemic vasculitis and thrombosis include widespread endothelial dysfunction resulting in a decreased production of thrombomodulin and other anticoagulant factors (Trifiletti, Scamardi, Bagnato, & Gaudio 2009); structural arterial injury including aneurysm formation and /or stenoses resulting in altered shear stress forces and flow dynamics (Brogan et al. 2002); and anti plasminogen antibodies compromising fibrinolysis (Berden et al. 2010). Our lack of clear understanding of the relative contribution of each of these possibilities however makes primary thrombosis prevention strategies challenging, with lack of suitable biomarkers that could define risk in routine clinical practice (Tomasson, Monach, & Merkel 2009). The only notable exception is for patients with Kawasaki disease with giant coronary artery aneurysms (>8mm), in whom combined use of antiplatelet therapy and anticoagulation is well established to reduce the risk of myocardial infarction (Sugahara et

al. 2008). Therefore in the absence of a clear mechanism to explain the thrombosis in vasculitis, it has been difficult to define a biomarker which could identify patients at risk of this complication. Consequently, clinical practice relating to this issue varies widely and published guidance mainly focuses on treatment of thrombosis after the event, and secondary prevention (Tomasson, Monach, & Merkel 2009).

### ***5.2.2 Thrombin generation assays***

Thrombin generation is the endpoint of a series of proteolytic reactions that start with the formation of tissue factor-activated factor VII (FVII) complex following vessel wall injury (figure 5-1)(Hemker et al. 2004; Van Veen et al. 2008). This results in the generation of small amounts of thrombin through activation of the prothrombinase complex, insufficient to cause full conversion of fibrinogen to fibrin but causing feedback activation of factor V (FV), factor VIII (FVIII) and factor XI (FXI) subsequently leading to a thrombin burst converting fibrinogen to fibrin (figure 5-1) (Gailani and Broze Jr 1991; Hemker and Beguin 1995; Van Veen, Gatt, & Makris 2008). Cell surfaces containing phosphatidylserine (PS) are considered to provide a template for these reactions and importantly are now thought to actively coordinate the process (Hoffman and Monroe 2001). Thrombin accelerates its own generation by positive feedback systems but also inhibits it through its interaction with thrombomodulin (Stassen et al. 2004; Van Veen, Gatt, & Makris 2008). By binding to this endothelial receptor, thrombin loses its procoagulant function and can then activate protein C. Activated protein C (APC) inhibits activated FVIII (FVIIIa) and activated FV (FVa) with protein S acting as a vital cofactor (Esmon and Schwarz 1995;Walker and Fay 1992). In

addition, antithrombin is a further major natural anticoagulant inhibiting thrombin generation (Lawson et al. 1993). Lastly, after the injured tissue is repaired, clot degradation occurs to allow sufficient blood flow (Vassalli et al. 1991). Both tissue-type plasminogen activator (tPA) and plasminogen bind to the fibrin fibres followed by the formation of plasmin that then lyses the clot (Vassalli, Sappino, & Belin 1991). A number of natural inhibitors will inhibit excess levels of plasmin:  $\alpha$ 1-antitrypsin,  $\alpha$ 2-antiplasmin, C1-inhibitor and  $\alpha$ 2-macroglobulin ( $\alpha$ 2M) (van Geffen and van Heerde 2012). By the continuous degradation of fibrin, new C-terminal lysine residues become available creating a growing surface for extra stimulation of the formation of plasmin (van Geffen & van Heerde 2012). This enhanced stimulation is inhibited by the activation of thrombin activatable fibrinolysis inhibitor (TAFI) (van Geffen & van Heerde 2012; van Tilburg et al. 2000).

Conventional coagulation tests, such as the prothrombin time (PT) and activated partial thromboplastin time (APTT), are not capable of assessing the whole coagulation system (Van Veen, Gatt, & Makris 2008). These tests use clot formation as their endpoint which occurs when only around 5% of all physiologically relevant thrombin is formed and also are insensitive to identifying prothrombotic states (Hemker & Beguin 1995; Rand et al. 1996; Van Veen, Gatt, & Makris 2008). Measurement of the thrombin generating capacity, however, captures the end result of the interaction between proteases and their inhibitors and is therefore potentially more useful as a reflection of a thrombotic (high thrombin generation) or bleeding diathesis phenotype (low thrombin generation) compared to traditional coagulation tests (Hemker, Al Dieri, & Beguin 2004; Van Veen, Gatt, & Makris 2008). Measurement of thrombin generation was initially described in 1953 and involved

timed subsampling of aliquots from clotting blood or plasma onto a solution of diluted bovine plasma (without  $\text{Ca}^{++}$ ) and assessing the thrombin concentration in the sample from the clotting time observed (Macfarlane and Biggs 1953). The method used was however laborious and subject to high variability. Hemker et al. were the first who reported the use of a chromogenic substrate to determine thrombin generation in plasma late 1980s (Hemker et al. 1986; Hemker et al. 1993). Subsampling onto a chromogenic thrombin substrate allowed automatic recording of the sampling time and semi-automatic measurement of the thrombin activities making it possible for several parallel experiments to be done (Hemker, Wielders, Kessels, & Beguin 1993; Van Veen, Gatt, & Makris 2008). This substrate used was split by thrombin as it was generated in the recalcified plasma and released p-nitroaniline that was then measured spectrophotometrically (Hemker, Wielders, Kessels, & Beguin 1993; Van Veen, Gatt, & Makris 2008). However there were certain limitations of this assay. The conversion of prothrombin to thrombin and subsequent feedback activation usually leads to mechanisms to control thrombin formation. One of these mechanisms involved, is the covalent binding of thrombin to  $\alpha_2\text{M}$  (Hemker et al. 2007; van Geffen & van Heerde 2012; Van Veen, Gatt, & Makris 2008; Wagenvoort et al. 2010). This interaction inhibits the activity of thrombin towards its natural substrates, but still allows the conversion of small, synthetic substrates leading to residual substrate conversion (Hemker, De Smedt, & Al Dieri 2007; Van Veen, Gatt, & Makris 2008; Wagenvoort, Deinum, Elg, & Hemker 2010). Therefore the final concentration of the thrombin- $\alpha_2\text{M}$  complex is proportional to the total thrombin generation (Hemker et al. 2000; van Geffen & van Heerde 2012; Van Veen, Gatt, & Makris 2008). Two methods were developed to overcome this issue: First, the residual activity in the thrombin generation curve was corrected by mathematical modelling and also

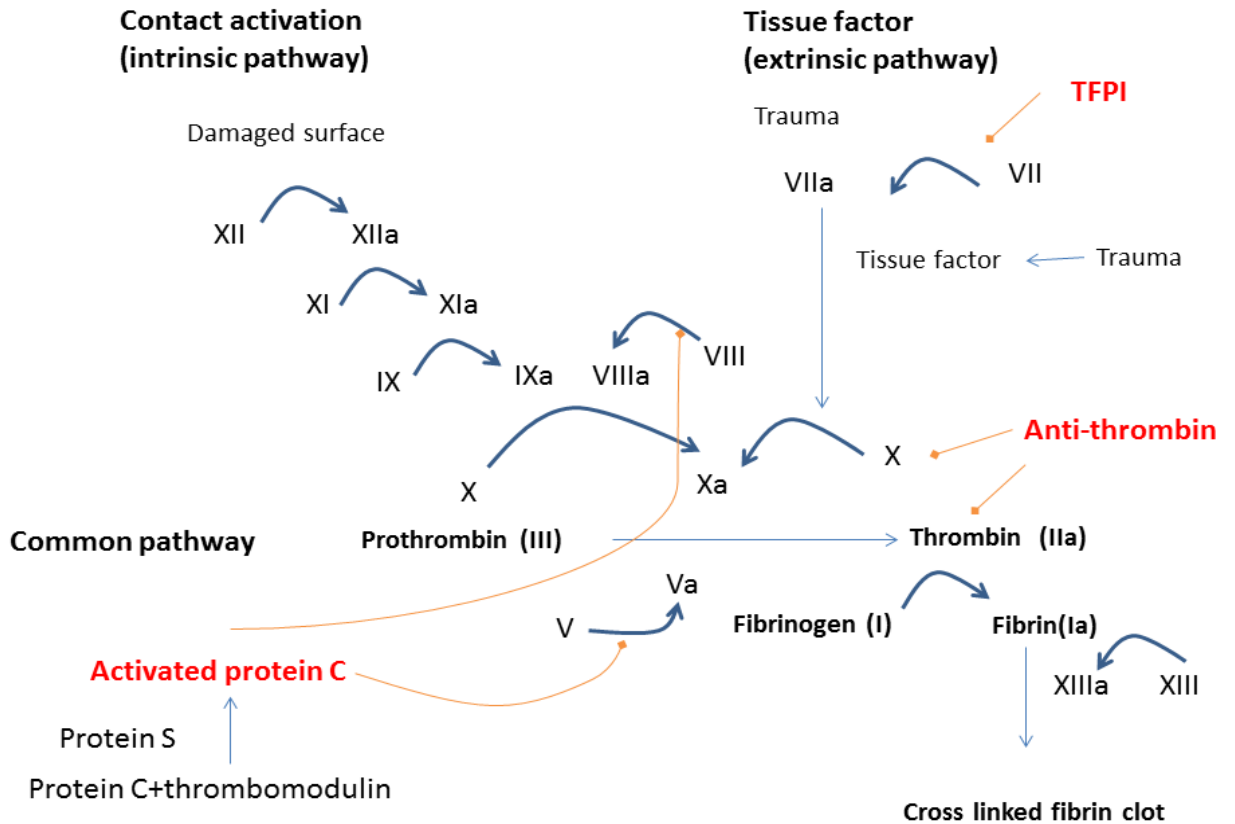
hydroxylamine or a metalloprotease were employed to remove the  $\alpha$ 2M activity (Rijkers et al. 1998; Hemker et al. 2000; van Geffen & van Heerde 2012; Van Veen, Gatt, & Makris 2008). Of note, another limitation of using chromogenic substrates is that these emit a signal with a wavelength in the visual spectrum (Hemker et al. 2000; van Geffen & van Heerde 2012; Van Veen, Gatt, & Makris 2008). Therefore defibrinated and platelet-poor plasma is required, because the change in turbidity during coagulation interferes with the emitted signal of the split product (Hemker et al. 2000; van Geffen & van Heerde 2012; Van Veen, Gatt, & Makris 2008).

The drawback of turbidity can fortunately be overcome by the use of fluorescent substrates that have since been developed (Hemker et al. 2000; van Geffen & van Heerde 2012; Van Veen, Gatt, & Makris 2008). Hemker et al. specifically developed a fluorescent substrate Z-Gly-Gly-Arg coupled to 7-amino-4-methylcoumarin (AMC) which has been subsequently widely used for thrombin generation measurements (Hemker et al. 2000). Upon cleavage by thrombin, this substrate releases the AMC fluorophore that has an excitation wavelength of 390 nm and an emission wavelength of 460 nm (Hemker et al. 2000). There were, however, limitations associated with the use of fluorogenic substrates that had to be overcome. Firstly, plasma absorbs a significant and variable amount of the light (van Geffen & van Heerde 2012). There is also variation between apparently normal plasma (van Geffen & van Heerde 2012). Using the available substrates, the proportionality between reaction velocity and product formation can not be maintained during the experiment and as a result, the same amount of thrombin activity causes a far greater increase of signal at the beginning of the experiment than towards the end (van Geffen & van Heerde 2012). Moreover, the readings

are very sensitive to the colour of the plasma, for instance even for minimal haemolysis (van Geffen & van Heerde 2012). These drawbacks of fluorescent methods have to be dealt with by continuous calibration of every sample (van Geffen & van Heerde 2012). In this technique, a fixed amount of constant thrombin activity is added to a parallel sample (van Geffen & van Heerde 2012). From the resulting curve, the calibration factor at any level of fluorescence is read and the exact thrombin concentration in the sample where thrombin generation is taking place can be calculated (van Geffen & van Heerde 2012).

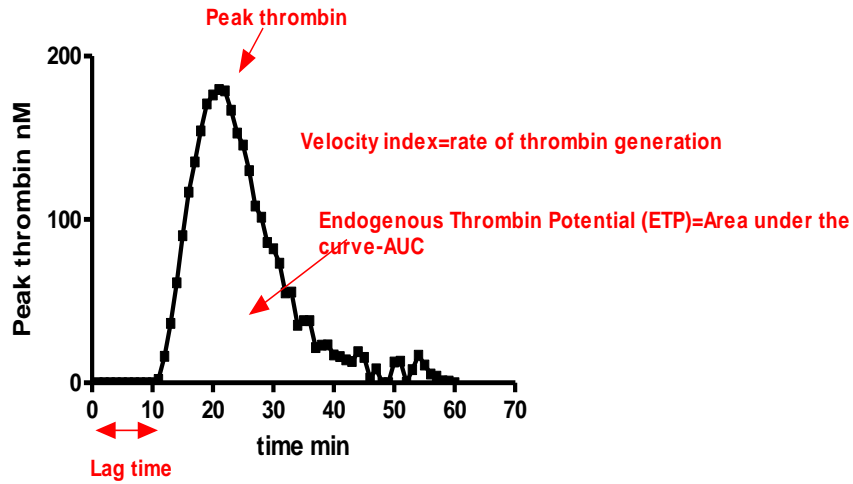
These modifications have led to standardisation of the technique, with good reproducibility and little variation thus permitting wider use of thrombin generation measurements in clinical studies (Hemker, Al Dieri, & Beguin 2004; Hemker et al. 2000; van Geffen & van Heerde 2012). The parameters measured include: (i) the lag time defined as the moment that the signal deviates by more than 2 standard deviations from the horizontal baseline; (ii) peak thrombin generation; (iii) velocity index which is defined as the rate of thrombin formation and (iv) the endogenous thrombin potential (ETP) equivalent to the area under the curve (Hemker, Al Dieri, & Beguin 2004; Hemker et al. 2000; van Geffen & van Heerde 2012). A typical trace is shown in figure 5-2. Thrombin generation measuring the cumulative effect of pro-thrombotic tendencies has since been used as a predictive parameter for thrombosis recurrence (Hemker, Al Dieri, & Beguin 2004; Hemker et al. 2000; van Geffen & van Heerde 2012). A number of previous studies have established that the presence of (prothrombin G20210A) mutation and hereditary deficiencies of protein C, protein S and antithrombin are all associated with increased thrombin generation (van Geffen & van Heerde 2012). Table 5-1 summarises the data of recent studies examining the relationship

between thrombin generation and hypercoagulable states, limited to studies using a fluorogenic thrombin generation method. Therefore thrombin generation as a global test for overall function of the plasmatic coagulation can be profitably applied to establish thrombotic tendencies in clinical practice.



*Figure 5-1: The coagulation cascade.*





**Figure 5-2: Thrombin generation assay curves.**

Measures recorded are (i) peak height, in nM thrombin, (ii) lag time (time to onset of thrombin generation), (iii) velocity index = rate of thrombin generation, and (iv) endogenous thrombin potential (ETP) referring to the area under the curve.

<b>Studies</b>	<b>Subjects</b>	<b>Plasma</b>	<b>Result</b>
Andresen et al. (2004)	24 patients with heritable thrombophilia (7 previous VTE) and 24 controls	PPP	Mean ETP in patients > controls. Patients with AT deficiency (n = 6) >upper limit normal
Ay et al. (2011)	1033 patients with malignancies. VTE occurred in 7.5%	PPP	Patients with elevated peak thrombin ( $\geq 611$ nM thrombin) had an increased risk of VTE
Castoldi et al. (2007)	302 individuals from unrelated families. 167 heterozygous (39 with previous thrombosis) and 3 homozygous for the F2 G20210A mutation vs. 132 non carriers (14 with previous thrombosis)	PPP	Significantly increased thrombin generation in patients with increased FII concentration due to the F2 G20210A mutation compared to non carriers and increased FII not associated with this mutation
Chaireti et al. (2009)	98 patients with the FV Leiden and 15 with the prothrombin mutation and an equal number of age- and gender-matched controls.	PPP	Prolonged time both for the initiation and termination of thrombin generation in patients with thrombosis
Dargaud et al. (2006)	71 normal subjects, 11 women on the COCP, 89 patients after first DVT	PPP	ETP >mean + 2SD of normal was associated with OR 40.5 in patients with congenital thrombophilia and OR 5.85 without thrombophilic makers

			for 1st DVT
Hezard et al. (2006)	169 patients with personal or family history of VTE	t-PRP	Significant APC resistance in patients with protein S deficiency and F5 R506Q compared to patients without heritable thrombophilia
Hezard et al. (2007)	102 patients with personal or family history of VTE	t-PRP	Significant APC resistance in patients with protein C/S deficiency and F5 R506Q compared to patients without heritable thrombophilia
Hron et al. (2006)	914 patients with 1st VTE of which 100 had a recurrence	PPP	Recurrence risk 6.5% if peak thrombin <400 nmol/l and 20% if peak thrombin >400 nmol/l
Lecompte et al. (2007)	40 patients with antiphospholipid antibodies (24 with thrombosis) and 19 controls	PRP	Significant activated protein C resistance with OR for thrombosis up to 7
Liestol et al. (2007)	81 patients with LA (52 APS, 34 on warfarin) and 91 controls	PPP	Thrombin generation in LA plasma is increased independent of warfarin use and the degree of APC appears to be associated with thrombotic events

Lutsey et al. (2009)	434 cases and 1004 controls.	PPP	Relative to the first quartile of peak thrombin generation, the odds ratio of VTE for those above the median was 1.74 (95% CI 1.28-2.37)
Sonnevi et al. (2008)	243 women with a first VTE. In women without factor V Leiden (n=117), samples analysed in the absence and in the presence of APC	PPP	Increase in ETP and peak height in the presence of APC correlated significantly with higher risk of recurrence.
Tappenden et al. (2007)	50 patients with VTE (56% idiopathic) and 31 controls	PPP/PRP/WB ± CTI	Thrombin generation was significantly increased in VTE patients in the WB assay only
van Hylckama Vlieg et al. (2007)	360 patients after a first VTE and 404 controls	PPP	OR 1:5 for 1st VTE

**Table 5-1: Studies exploring the relation between thrombin generation and hypercoagulable states, limited to those using a fluorogenic method.**

*Adapted from van Veen et al. (2008). VTE= venous thromboembolism; AT=anti-thrombin; LA=lupus anticoagulant; FVIII: C=FVIII coagulant activity; COCP=combined oral contraceptive pill; DVT= deep vein thrombosis; APS=antiphospholipid syndrome; PPP= platelet poor plasma; (ft-) PRP, (frozen-thawed-) platelet rich plasma; WB= whole blood; CTI= corn trypsin inhibitor; ETP= endogenous thrombin potential; OR= odds ratio; APC= activated protein C; CI=confidence intervals.*

### ***5.2.3 Pro-coagulant microparticles***

As described in chapter 4 MPs are plasma membrane vesicles released from cells during activation and apoptosis (Morel et al. 2006; Piccin, Murphy, & Smith 2007; Sabatier et al. 2009). These circulating MPs are known to alter crucial biologic functions by eliciting inflammation, oxidative stress or apoptosis and thrombosis (Morel et al. 2006; Piccin, Murphy, & Smith 2007; Sabatier et al. 2009). Normal haemostasis is characterised by a dynamic balance between the procoagulant and anticoagulant components of the coagulation cascade. MPs may potentially shift this equilibrium in favor of a procoagulant phenotype (Morel et al. 2006; Piccin, Murphy, & Smith 2007; Sabatier et al. 2009). The currently proposed prothrombotic properties of MPs include: the provision of a large phosphatidylserine (PS) surface area for the assembly and activation of coagulation enzyme complexes; expression of tissue factor (TF) (Morel et al. 2006; Piccin, Murphy, & Smith 2007; Sabatier et al. 2009); and the ability to directly activate platelets in the immediate vicinity of thrombus formation (Morel et al. 2006; Piccin, Murphy, & Smith 2007; Sabatier et al. 2009). Under physiological conditions, very little TF is present in circulation; however, during inflammatory states, endothelial cells and monocytes are able to synthesize TF and shed it in microparticles (Giesen et al. 1999; Shet et al. 2003; Zwicker et al. 2011). In monocytes, TF-bearing microparticles arise from membrane regions that are rich in lipid-raft domains which endow the microparticles with other raft-associated proteins such as PSGL-1, the primary vascular counter-receptor for P-selectin (Falati et al. 2003). When platelets are activated they express P-selectin, allowing microparticles to attach to and fuse with the

platelets by a phosphatidylserine-dependent mechanism (Del Conde et al. 2005a; Del Conde, Shrimpton, Thiagarajan, & López 2005b). This fusion further increases the procoagulant activity of the TF (Del Conde, Shrimpton, Thiagarajan, & López 2005b). Additionally, a recent study showed that in vitro activated neutrophils generate microparticles that expose the integrin CD11b/CD18 in an active conformation capable of binding GP Iba and activating platelets (Pluskota et al. 2008).

In systemic vasculitis the presence of elevated MP during active disease states has been previously established by multiple studies (Brogan et al. 2004; Clarke et al. 2010; Erdbruegger et al. 2008). However the majority of these have focused on identification and enumeration of circulating MP in plasma without examining their functional properties particularly their pro-coagulant potential (Brogan et al. 2004; Clarke et al. 2010; Erdbruegger et al. 2008). A modification of the traditional thrombin generation assay described above could provide a reproducible and precise method of directly measuring the MP procoagulant properties and allow us to define the MP contribution to thrombosis in systemic vasculitis and other vascular disorders.

### **5.3 Aims**

Children with active vasculitis have been previously shown to have increased annexin V+ circulating MPs of endothelial and platelet origin. In this chapter the hypothesis that these MP are prothrombotic and could differentiate patients with and without clinical thrombotic complications is explored. The aim of this study was thus to define the pro-coagulant

activity of plasma MPs and in relation to clinical thrombotic events in children with systemic vasculitis.

## **5.4 Methods**

### ***5.4.1 Subjects, classification of vasculitic syndromes and relation to disease activity***

Children with primary systemic vasculitis attending a single centre (Great Ormond Street Hospital, London) between October 2008 and June 2010 were studied. Inclusion criteria were: age <18 years, a diagnosis of primary systemic vasculitis confirmed by histopathologic and/or arteriographic assessment and exclusion of secondary causes of the vasculitis (infection, connective tissue disease, or malignancy). Control samples were obtained from healthy age- and sex-matched children. The study was approved by the Institutional Ethics Committee (project ethics number 04/Q0508/117). Fully informed written consent was obtained from all subjects studied.

The vasculitis subtype was classified using the new European League Against Rheumatism/Paediatric Rheumatology International Trials Organisation/Paediatric Rheumatology European Society (EULAR/PRINTO/PRES) classification criteria for paediatric vasculitides by Ozen et al. for polyarteritis nodosa (PAN) and GPA (formerly known as Wegener's granulomatosis; WG) (Ozen et al. 2010). Definitions for Kawasaki Disease (KD), Behçets Disease (BD), and Churg Strauss syndrome (CSS) are not included in this classification

scheme. KD was thus defined as patients fulfilling at least 5/6 American Heart Association criteria (Newburger et al. 2004); BD was defined based on the International Study Group for BD published set of diagnostic criteria (International study group for Behçets disease, 1990). CSS was defined using the Chapel Hill consensus criteria (Jennette et al. 1994). Patients with unclassified vasculitis had histologically proven vasculitis but did not satisfy criteria for any one category.

Disease activity was assessed using a modified paediatric Birmingham Vasculitis Activity Score (BVAS) incorporating age specific laboratory reference ranges, as previously described (see appendix 6) (Brogan et al. 2004; Luqmani et al. 1994). Active vasculitis was defined as a score greater than zero for BVAS items attributable to vasculitis that newly appeared or worsened during the preceding 4 weeks and for which other causes such as infection were excluded (Brogan et al. 2004; Luqmani et al. 1994). The following routine laboratory markers providing adjunctive information related to vasculitis activity were determined: erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) levels. Thrombosis was classified based on clinical events and confirmed by vascular ultrasound, computed tomography, magnetic resonance or catheter angiography. Samples were taken at the onset of thrombotic events. Supporting evidence of ongoing endothelial injury was provided by enumeration of CECs as described previously in chapter 3.



#### **5.4.2 Assessment of routine prothrombotic risk factors**

Conventional risk factors for thrombosis were assayed routinely by the haematology clinical laboratory at Great Ormond Street Hospital. These included assessments for deficiency of protein C, protein S, antithrombin III, presence of anticardiolipin antibodies and lupus anticoagulant, genetic mutations of factor V Leiden gene, prothrombin gene *G20210* and methylene tetrahydrofolate reductase (*MTHFR C677T*). Measurement of antithrombin III was based on a functional chromogenic assay performed using Siemens reagents supplied by Sysmex. Protein C was measured using a functional chromogenic assay performed with Unimate reagents supplied by Pathway Diagnostics. APC resistance was based on a Factor V dependent prothrombin activator isolated from snake venom using reagents supplied by Hart Biological. Protein S was assayed using an immunoturbidometric assay performed using reagents Stago diagnostic reagents. Dilute Russel Viper Venom Test (DRVVT) was assayed using Unitest reagents supplied by Pathway Diagnostics. All above assays were performed on a Sysmex CA1500 coagulation analyser. The titres of anticardiolipin antibodies were measured using an ELISA test AESKULISA CardioliPinGM supplied by Grifols.

Testing for the identification of the factor V Leiden (*FVL*) mutation, the pro-thrombin gene mutation (*G20210*), and the methylenetetrahydrofolatereductase (*MTHFR*) gene mutation was performed in the clinical haematology lab of Great Ormond Street Hospital using allelic discrimination by fast chemistry real time PCR according to published methods (Sebastian, Voetsch, Stone, Romay-Penabad, Lo, Allen, Davis Jr, Hoffman, McCune, & St Clair 2007).

### ***5.4.3 Cellular microparticle identification by flow cytometry***

MPs were identified by flow cytometry as previously described in chapter 4. MPs sedimented from 200µl of PPP after centrifugation at 15000g for 60 min were re-suspended in Annexin V binding buffer (BD PharMingen, Oxford, United Kingdom) and stained with annexin V conjugated with fluorescein isothiocyanate (FITC; BD PharMingen) or annexin V-phycoerythrin (PE; BD PharMingen, UK). For measuring EMP, PMP, NMP and TF staining samples were additionally incubated with mouse (PE)-labeled anti-human CD62e (Clone 68-5H11, BD PharMingen, UK), mouse TRI-COLOR labeled anti-human CD41 (Clone VIPL3, Caltag laboratories, UK), PE-conjugated anti-human CD11b activation epitope (clone CBRM1/5, Biolegend, UK), and mouse FITC-conjugated anti-human TF (clone VD8, American Diagnostica, USA) respectively as well as the relevant isotype controls (as per manufacturer recommendation) for 20 minutes at room temperature. Samples were analyzed with a FACSCalibur flow cytometer (Becton Dickinson).

### ***5.4.4 Thrombin generation assay (TGA)***

The TGA measures the amount of active thrombin produced in plasma or whole blood after re-calcification by monitoring the conversion of a fluorogenic substrate cleaved by thrombin as the latter is generated (Hemker et al. 2008). Direct calculation of molecular concentrations of thrombin are possible by comparison to a concurrently run calibrator (see appendix 7). For the thrombin generation assay MPs sedimented from PPP as above were re-suspended in 200 µL of control microparticle-free plasma (MPFP): MP free plasma prepared after MP sedimentation from approximately 50-60 mL plasma from adult healthy volunteers,

with 30 µg/ml of corn trypsin inhibitor (Sigma) added to prevent potential in vitro contact activation of the coagulation cascade. Subsequently 40 µL of MPs suspended in control MPFP was added to the plate well, followed by 50 µL of calcium-fluorogenic substrate (0.5 mM/L of Z-G-G-R-AMC and 7.5 mM/L of calcium final reagent concentrations, Pathway Diagnostics). No exogenous TF or phospholipids were added at this stage to ensure that the thrombin generated was solely dependent on MP related coagulant activity. The thrombin generated was measured by fluorogenic excitation/ emission at 360/460 nm at 1 minute time intervals for 90 minutes against a standard thrombin calibrator (Pathway Diagnostics) in an Optima fluorescence plate reader (BMG). Measures recorded were: (i) height of peak thrombin (nM); (ii) lag time (time to onset of thrombin generation); (iii) velocity index = rate of thrombin generation; and (iv) endogenous thrombin potential (ETP) = the area under the curve (Figure 5-2).

#### ***5.4.5 In vitro microparticle mediated thrombin generation***

A number of potent stimulants were used to induce in vitro MP formation in whole blood (5 µg/ml of LPS derived from *E. Coli*, O55:B5 stimulation for 4 hours; TNF- $\alpha$  100 ng/ml for 5 hours; and calcium A23187 10 nM for 1 hour). Unstimulated samples were used as controls. PPP was prepared from these samples following centrifugation at 5000g twice. MP presence was confirmed with flow cytometry as previously described. As EMP normally represent a sparse population of MP, whole blood stimulated with LPS was also spiked with EMPs pelleted from supernatants derived from HUVEC stimulated with TNF- $\alpha$  (100 ng/ml for 24 hours). MP pellets derived from thus stimulated and control unstimulated samples were then

resuspended in MPFP and processed for TGA as described in 5.4.4. All samples were analysed in triplicate to establish the reproducibility of the technique.

#### ***5.4.6 Thrombin generation capacity of plasma following removal of MPs by filtration***

PPP samples obtained from whole blood stimulated with lipopolysaccharide (LPS derived from *E. Coli*, O55:B5, Sigma) at 5 µg/ml for 4 hours to induce MP formation spiked with EMP (pelleted from supernatants derived from HUVEC stimulated with TNF-α at 100 ng/ml for 24 hours) were filtered through a 0.2 µm, low protein binding filter to remove MPs. MPs were recovered from filters by rinsing with an equal volume of MPFP. The thrombin generating capacity of filtered and unfiltered samples was assessed.

#### ***5.4.7 Relative contribution of PS and tissue factor to MP mediated thrombin generation***

To investigate the relative contribution of PS and TF to MP mediated thrombin generation, MPs isolated from LPS stimulated whole blood (5 µg/ml for 4 hours) spiked with EMP (pelleted from supernatants derived from HUVEC stimulated with TNF-α at 100 ng/ml for 24 hours) were pre-incubated with increasing concentrations of purified recombinant AnV protein (BD Pharmingen) to inhibit PS; or a blocking monoclonal antibody against TF (epitope areas of amino acids 1-25 of TF, American Diagnostica) or control purified IgG1 antibody (Biolegend) at 37<sup>0</sup> C for 20 minutes. MPs pre-treated in these experiments were then re-suspended in control MPFP (prepared as described above) and the thrombin

generating capacity was determined. Dose response curves were thus obtained and allowed optimal concentrations for these blocking reagents to be established:  $1\mu\text{g}/10^6$  MPs for AnV; and  $20\ \mu\text{g}/10^6$  MPs for TF. These concentrations were then used in similar inhibition experiments using MP derived from vasculitis and control patient samples.

#### ***5.4.8 Differential contributions of monocyte and platelet-derived microparticles towards thrombin generation and tissue factor pro-coagulant activity***

In order to examine the specific contributions of monocyte and platelet-derived MPs to thrombin generation MPs were prepared from stimulated human platelets and monocytes as described in chapter 4. MPs were then resuspended at a concentration of  $10^6$  /ml in MPFP and then compared as for their ability to initiate thrombin generation.

FXa generation per MP was also measured by chromogenic substrate cleavage and referenced to a standard curve of lipidated recombinant human TF (TF activity kit, American Diagnostica). In brief PPP samples prepared as above were mixed with human factor VIIa and human factor X and incubated at  $37^{\circ}\text{C}$  during which time the tissue factor/factor VIIa complex is formed and the complex converts the human factor X to Factor Xa. The Factor Xa generation was measured by chromogenic substrate cleavage (spectrozyme FXa that is highly specific to factor Xa) and referenced to a standard curve of lipidated recombinant human TF. Solution absorbance was read at 405 nm in an Optima plate reader (BMG).

These experiments comparing the differential contributions of monocyte and platelet MP to thrombin generation and TF activity were repeated with MPs pre-incubated with anti-TF antibody ( $20 \mu\text{g}/10^6$  MP) or an isotype-matched control IgG for 15 min at  $37^0$  C.

## **5.5 Statistical analysis**

All experiments were performed at least in triplicate, and values are presented as mean  $\pm$  SEM unless otherwise specified. Statistical differences for in vitro experiments between groups were determined by two-way ANOVA, followed by unpaired two-tailed t test. Patient demographics and comparisons between groups were summarized as median and range unless otherwise specified. The Kruskal-Wallis test was used to examine overall differences in experimental laboratory markers between the study groups. Following this, statistical differences between groups were determined by Mann-Whitney U test. Associations between cumulative MP counts and peak thrombin generated were assessed using Spearman's rank correlation coefficient. Receiver operator characteristic (ROC) curves, sensitivity, specificity, positive and negative predictive values, and likelihood ratios were calculated to examine the diagnostic test characteristics of peak thrombin for thrombosis in active vasculitis. ROC analysis was reported as area under the curve (AUC) and 95% confidence intervals (CI). P-values of less than 0.05 (two sided) were regarded as significant. Statistical analysis was performed using SPSS version 17.

## 5.6 Results

### ***5.6.1 Patients and controls***

Twenty five children, 8 male, with a median age of 10 years (range 1.2-16.1) at diagnosis with systemic vasculitis were studied. There were 15/25 children with active vasculitis (PAN n=7; KD n= 3; WG n=3; CSS n=1; BD n=1) with BVAS 7/63 (5-20/63), ESR 56.5 (40–159) mm/h, CRP 45 (10–270) mg/L and CECs 240 (112-1600) cells/ml; and 10 children with inactive vasculitis (PAN n=3, WG n=4, unclassified n=2, CSS n=1) BVAS=0/63 in all, ESR 5 (5–10) mm/h, CRP 7 (5–15) mg/ L and CECs 46, range 40-112 cells/ml. Five children with active vasculitis were on corticosteroids and one child had received one dose of intravenous (i.v.) cyclophosphamide. Six children with inactive vasculitis were on oral corticosteroids and i.v. cyclophosphamide; two were receiving azathioprine and two were on mycophenolate mofetil.

Of those patients with active vasculitis, 7/15 presented with thrombotic events: cerebral arterial infarction n=2 (both with PAN); cerebral sinovenous thrombosis n=1 (BD); myocardial infarction n=1 (KD); digital infarction n=2 (both with PAN); thrombotic peripheral gangrene n=1 (WG). Two of these children whose disease was complicated by thromboses (a patient with PAN and cerebral arterial infarction and the patient with WG and peripheral skin gangrene) were also studied following treatment of vasculitis after remission of vasculitis had been induced.

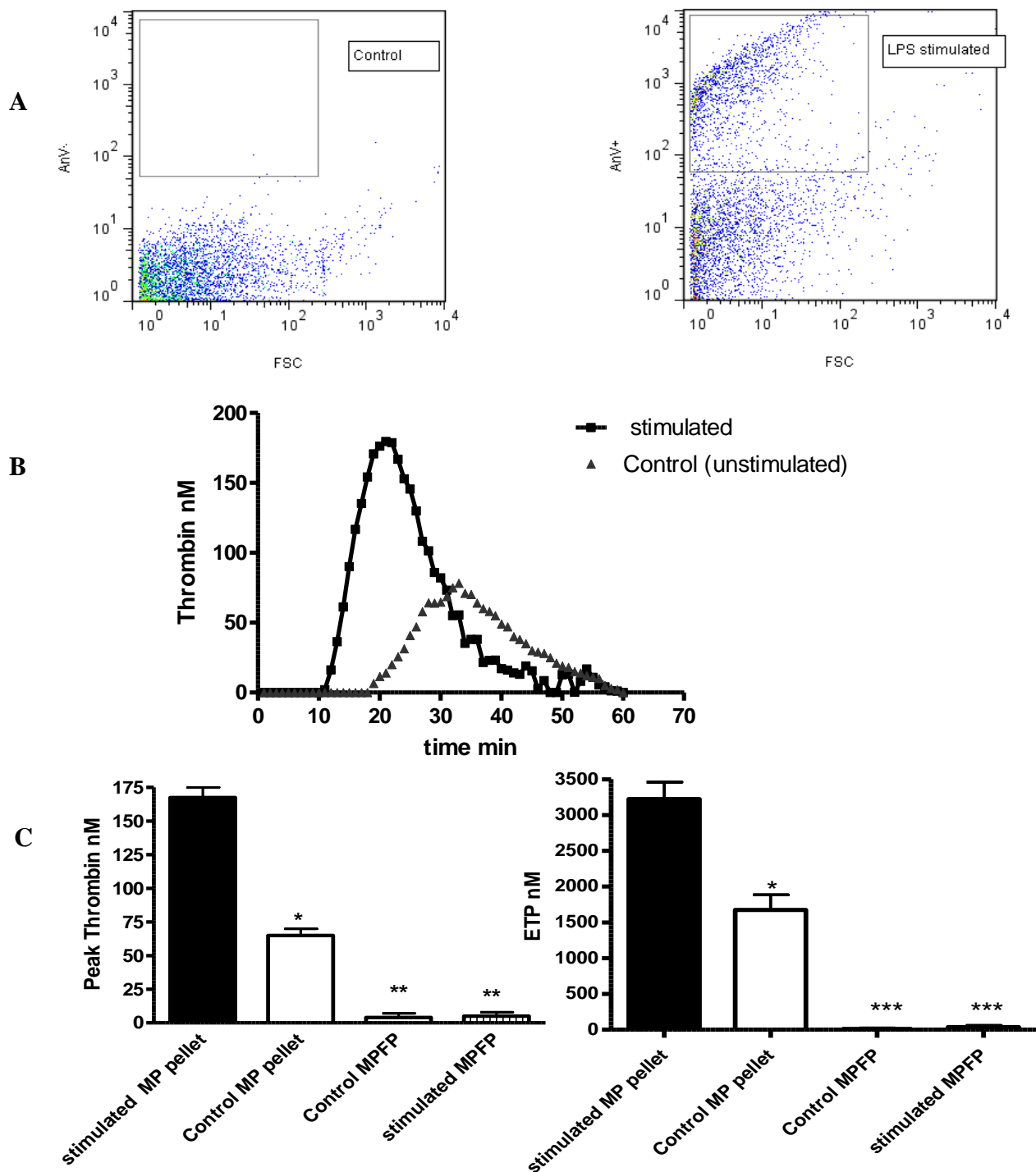
Five children out of 25 (including 2 who had a thrombotic event) were found to be heterozygous for MTHFR C677T mutations. The rest of the screen for conventional pro-thrombotic risk factors was negative for all remaining patients. None of the children were receiving anti-coagulant therapy at the time of sampling. All children with KD were on high “anti-inflammatory” dose aspirin 40 mg/kg/day. There were 10 paediatric healthy controls, 3 male, median age 9.8 years (2-16 years) with a median CEC count of 24 cells/ml (range 0-80 cells/ml).

### ***5.6.2 LPS stimulation of whole blood results in increased MP-mediated thrombin generation***

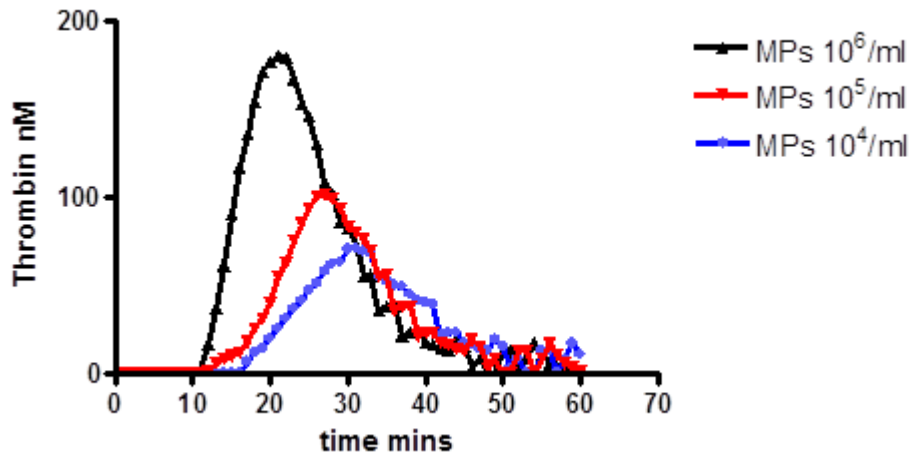
The ability to detect the MP mediated thrombin generating capacity by using a potent inflammatory stimulus was initially confirmed. MP suspensions from LPS stimulated whole blood (5 µg/ml for 4 hours) contained significantly higher number of circulating total Annexin V+ MPs 1200 (range 800-1400)  $\times 10^3$ /ml compared to healthy control blood 200 (50-250)  $\times 10^3$ /ml,  $p=0.001$  (figure 5-3A). Representative thrombin generation curves for LPS-stimulated and un-stimulated samples are shown in figure 5-3B. These MP suspensions also exhibited significantly greater MP-related peak thrombin median 167 (range 160-175 nM) and ETP median 3286 (range 2656-3661) nM  $\times$  min compared to healthy control blood not treated with LPS: median peak thrombin 65, range 60-70 nM,  $p=0.02$ ; and median ETP 1528 (range 1376-2269) nM  $\times$  min,  $p=0.02$  ( $n=3$ ; figure 5-3B). There additionally was a shorter lag time median 11 min (range 10-15 min) and increased velocity index 17 nM/min



(range 15-25 nM/min) for MP suspensions derived from stimulated whole blood compared to controls lag time 17 min (range 15-21),  $p=0.001$  and velocity index 7 nM/min (5-12 nM/min),  $p=0.002$ . For control MPFP alone (no MPs added) there was negligible contribution to thrombin generation (Figure 5-3C). The average coefficient of variation for these measurements was 5.2% (range 1.7-8.2%) suggesting a good reproducibility of the technique. Increasing concentrations of plasma MPs resulted in shorter lag time, higher peak of thrombin; increased velocity index and enhanced ETP (figure 5-4). Similar results were obtained with other stimulants (TNF- $\alpha$  and calcium A23187) and also when LPS stimulated whole blood was spiked with EMP harvested from the supernatants of HUVEC stimulated with TNF $\alpha$  (100ng/ml for 24 hours).



**Figure 5-3: LPS stimulation of whole blood induces microparticle formation with thrombin generating capacity.** (A) Microparticle suspensions from LPS stimulated whole blood contained significantly higher number of total AnnexinV+ MPs compared to control blood,  $p=0.001$ . (B) Representative thrombin generation curves for LPS-stimulated and unstimulated samples. (C) Microparticle suspensions from LPS stimulated whole blood exhibited significantly greater MP-related peak thrombin compared to healthy control blood,  $p=0.02$  ( $n=3$ ). MPFP=Microparticle free plasma. \*\*\* $P<0.005$  with student  $t$  test.

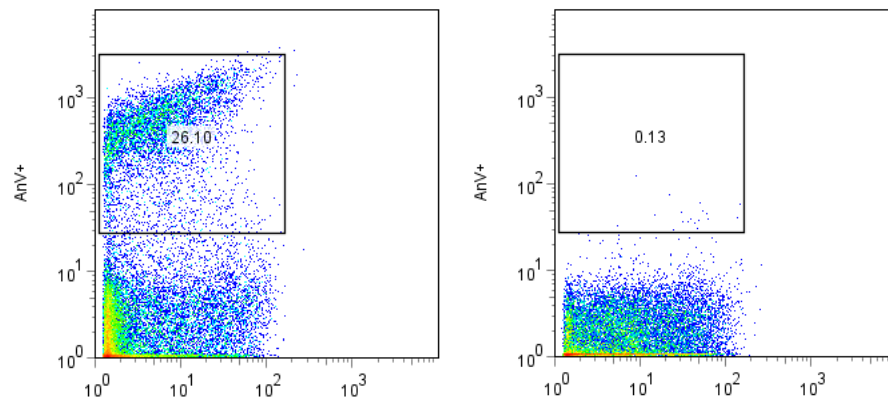


*Figure 5-4: Increasing concentrations of plasma microparticles resulted in shorter lag time; higher peak of thrombin; increased velocity index and enhanced endogenous thrombin potential.*

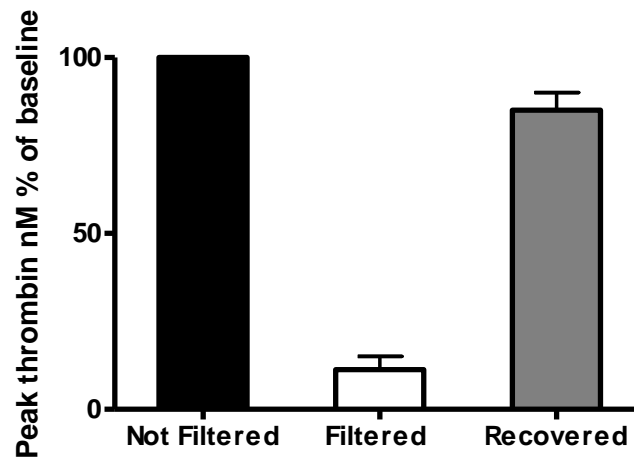
### ***5.6.3 Filtration of plasma removes MPs and abrogates MP-mediated thrombin generation***

Filtration of MP samples through a 0.2µm filter resulted in removal of the majority > 95% of circulating MPs from a count of 1300 (SEM 200) x10<sup>3</sup> /ml prior to filtration to 50 (SEM 20) x10<sup>3</sup> /ml following filtration (Figure 5-5 A). There was also significant reduction of the thrombin generating capacity of the sample down to 10% (SEM 2.5%) of the baseline thrombin generating capacity (Figure 5-5 B). MPs rinsed off the filter and reconstituted in 200µl of MPFP prepared as described above recovered their thrombin generation potential 95% (SEM 2.3%) of baseline peak thrombin (Figure 5-5B).

**A**



**B**



**Figure 5-5: Filtration of plasma removes microparticles (MPs) and abrogates MP-mediated thrombin generation.**

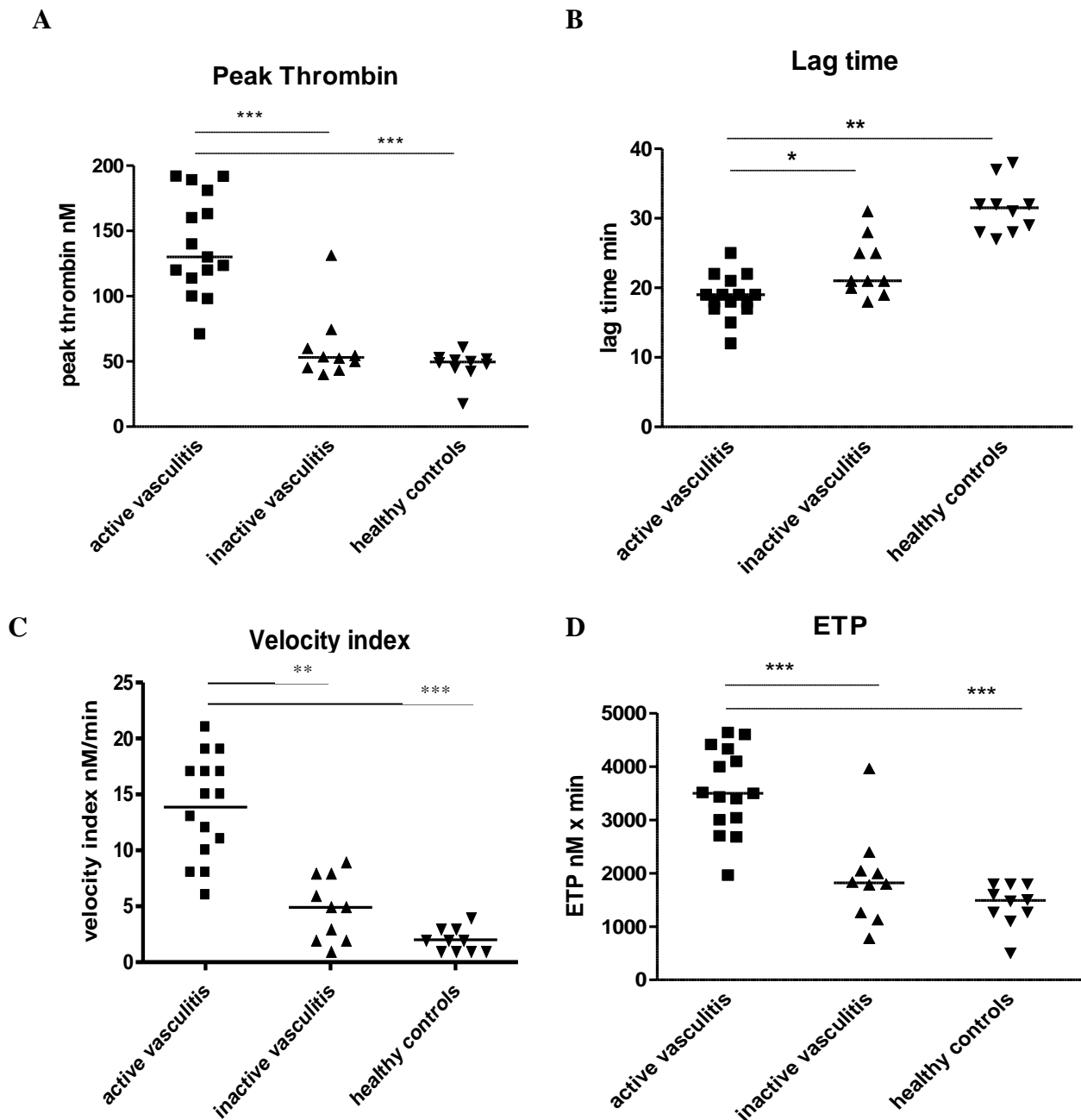
Filtration of plasma through a 0.2  $\mu\text{m}$  pore size filter abrogated thrombin generation by removing the majority of circulating microparticles (MP). The thrombin generating capacity of these MP could be almost completely recovered by rinsing these off the filter.

#### ***5.6.4 MP- mediated thrombin generation is increased in active vasculitis patients with thrombotic complications***

Children with active vasculitis exhibited a significantly higher peak thrombin of 145.85 (80.4-189.2) nM compared to the children with inactive vasculitis, median peak thrombin 53.6 (45-131.4) nM,  $p=0.002$ ; and healthy controls, peak thrombin 45 (24-60) nM,  $p=0.001$ ; Figure 5-6 A. For children with active vasculitis there was in addition a shorter lag time of median 18 minutes (12-18); higher velocity index median 15 nM/min (5.7-22); and higher median ETP of 3500 (1963-4639) nM x min compared to children with inactive vasculitis with lag time median 23 minutes (18-31),  $p=0.02$ ; velocity index median 2.9 nM/min (1.6-8.9),  $p=0.0001$ ; median ETP 1818 (783-3963) nM x min,  $p=0.0005$ ; and healthy controls with median lag time 32 minutes (27-38),  $p=0.002$ ; median velocity index 1.5 nM/min (0.06-2.2),  $p=0.0001$ ; median ETP 1373 (501-1800) nM x min,  $p=0.0005$ ; (Figure 5-6 B, C and D). The peak thrombin generated correlated significantly with the total number of plasma An V+ MPs:  $r_s=0.83$ ,  $p=0.0001$  (Figure 5-7).

Children with active vasculitis and thrombotic events ( $n=7/15$ ) had significantly higher peak thrombin, median 160 nM (113.9-191.9),  $p=0.0184$  compared to children with active vasculitis without thromboses, median 91.5 nM (80.4-130) (Figure 5-8). There were no significant differences between children with thrombosis in factor VIII levels 240 (110–320) IU/dL, fibrinogen 3.7 (3.1–7.2) g/L, platelet count 520 (420–650)  $\times 10^9/L$ , white cell count 16 (14–18)  $\times 10^9/L$  and those without thrombosis factor VIII 210 (144– 404) IU/dL,  $p = 0.7$ ,

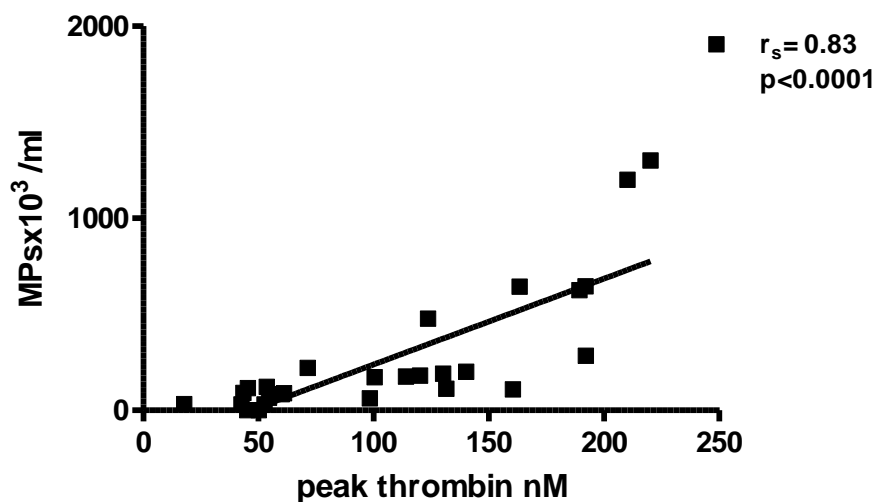
fibrinogen 3.4 (3.1–6.6) g /L, p = 0.3, platelet count 490 (400–620) x 10<sup>9</sup>/L, p = 0.6, white cell count 15 (13–18) x 10<sup>9</sup>/L, p = 0.7.



**Figure 5-6: Microparticle mediated thrombin generation in children with systemic vasculitis.**

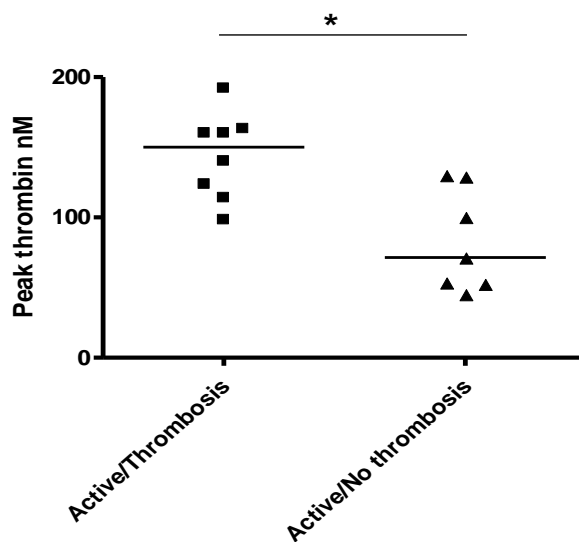
(A) Children with active vasculitis exhibited a significantly higher peak thrombin compared to the children with inactive vasculitis,  $p=0.002$ ; and healthy controls,  $p=0.001$ .

(B, C and D) For children with active vasculitis there was in addition a shorter lag time, higher velocity index and higher median endogenous thrombin potential (ETP) compared to children with inactive vasculitis and healthy controls.  $***P < 0.0005$  with Mann Whitney U test.



*Figure 5-7: The peak thrombin generated correlated significantly with the total number of plasma Annexin V+ MPs:  $r_s=0.83$ ,  $p=0.0001$ .*

MP induced thrombin generation in patients with thrombotic events



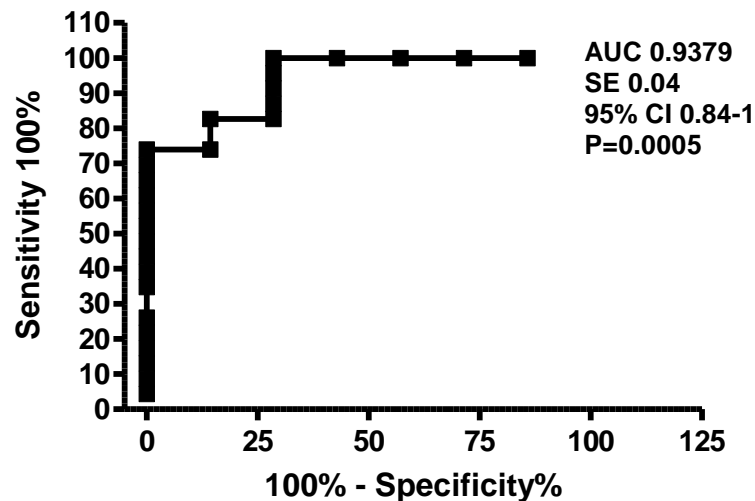
*Figure 5-8: Microparticle mediated thrombin generation in children with systemic vasculitis and thromboses.*

Children with active vasculitis and thrombotic events ( $n=7/15$ ) had significantly higher peak thrombin, median 160 nM (113.9-191.9 nM) compared to children with active vasculitis without thromboses, median 91.5 nM (80.4-130 nM),  $p=0.0184$ . \* $P<0.05$  by Mann Whitney U test.



### ***5.6.5 MP-mediated peak thrombin generation differentiates vasculitis patients with and without thrombosis***

ROC curve analysis of peak thrombin as a diagnostic test for thrombosis in children with systemic vasculitis was significant with an AUC of 0.93, SE 0.04 with 95% CI of 0.845-1.000,  $p=0.0005$  (figure 5-9). A cut-off peak thrombin  $>142.5\text{nM}$  with specificity of 95.65 % (95% CI of 78.05-99.89%) and sensitivity of 71.43% (95% CI of 29.04-96.73%) resulted in a positive likelihood ratio of 16.43 and negative likelihood ratio of 0.29 for thrombosis in children with systemic vasculitis with a positive predictive value of 94% (95% CI 85-98%). Table 5-2 summarises the diagnostic test characteristics of MP-mediated peak thrombin generation for thrombosis complicating active systemic vasculitis.



***Figure 5-9: Peak thrombin nM as a diagnostic test for thrombosis in childhood systemic vasculitis.***

*Receiver operator curve (ROC) analysis of peak thrombin as a diagnostic test for thrombosis in children with systemic vasculitis was significant with an area under the curve (AUC) of 0.93 with 95% CI of 0.845-1.000,  $p=0.0005$ .*

Peak thrombin nM	Sensitivity	95% CI	Specificity	95% CI	LR+	LR-	PPV	NNV
> 28.85	1.000	0.590-1.000	0.04348	0.001-0.2195	1.05	0	51	100
> 41.25	1.000	0.590-1.000	0.08696	0.010-0.2804	1.10	0	52	100
> 42.95	1.000	0.590 - 1.000	0.1304	0.02775 - 0.3359	1.15	0	53.48	100
> 44.25	1.000	0.590 - 1.000	0.1739	0.04951 -0.3878	1.21	0	54.64	100
> 45.20	1.000	0.590 - 1.000	0.2174	0.07460 -0.4370	1.28	0	55.87	100
> 47.65	1.000	0.590 - 1.000	0.2609	0.1023-0.4840	1.35	0	57.47	100
> 51.30	1.000	0.590 - 1.000	0.3478	0.1638-0.5727	1.53	0	60.61	100
> 53.10	1.000	0.590 - 1.000	0.3913	0.1971-0.6146	1.64	0	62.11	100
> 54.05	1.000	0.590-1.000	0.4348	0.2319-0.6551	1.77	0	63.69	100
> 57.25	1.000	0.590-1.000	0.4783	0.2682-0.6941	1.92	0	65.79	100
> 60.50	1.000	0.590 - 1.000	0.5217	0.3059-0.7318	2.09	0	67.57	100
> 66.15	1.000	0.590-1.000	0.5652	0.3449-0.7681	2.30	0	69.44	100
> 72.90	1.000	0.590 - 1.000	0.6087	0.3854-0.8029	2.56	0	71.43	100
> 86.35	1.000	0.590 - 1.000	0.6522	0.4273-0.8362	2.88	0	74.07	100
> 99.25	1.000	0.590-1.000	0.6957	0.4708-0.8679	3.29	0	76.34	100
> 107.1	1.000	0.590-1.000	0.7391	0.5160-0.8977	3.83	0	78.74	100
> 117.0	0.8571	0.421-0.996	0.7391	0.5160-0.8977	3.29	0.19	75.89	82.95
> 121.8	0.8571	0.421-0.996	0.8261	0.6122 - 0.9505	4.93	0.17	82.52	84.54
> 126.8	0.7143	0.290-0.963	0.8261	0.6122 - 0.9505	4.11	0.34	79.78	73.87
> 130.7	0.7143	0.290-0.963	0.8696	0.6641-0.9722	5.48	0.32	83.53	74.78
> 135.7	0.7143	0.290-0.963	0.9130	0.7196 - 0.9893	8.21	0.31	88.75	75.83
> 142.5	0.7143	0.290-0.963	0.9565	0.7805 - 0.9989	16.43	0.29	93.42	76.61
> 152.5	0.7143	0.290-0.963	1.000	0.8518 - 1.000	Infinity	0.28	100	77.52
> 161.5	0.5714	0.184-	1.000	0.8518 -	Infinity	0.42	100	69.63

		0.901		1.000				
> 172.1	0.4286	0.098-0.815	1.000	0.8518 - 1.000	Infinity	0.57	100	63.69
> 186.5	0.2857	0.036-0.709	1.000	0.8518 - 1.000	Infinity	0.71	100	58.14
> 192.0	0.1429	0.036 - 0.578	1.000	0.8518 - 1.000	Infinity	0.85	100	53.76

**Table 5-2: Test characteristics of microparticle peak thrombin generation for diagnosis of thrombosis in children with systemic vasculitis.**

*CI= confidence intervals; LR+=Likelihood ratio for a positive test; LR- =likelihood ratio for a negative test; PPV=positive predictive value; NPV=negative predictive value.*

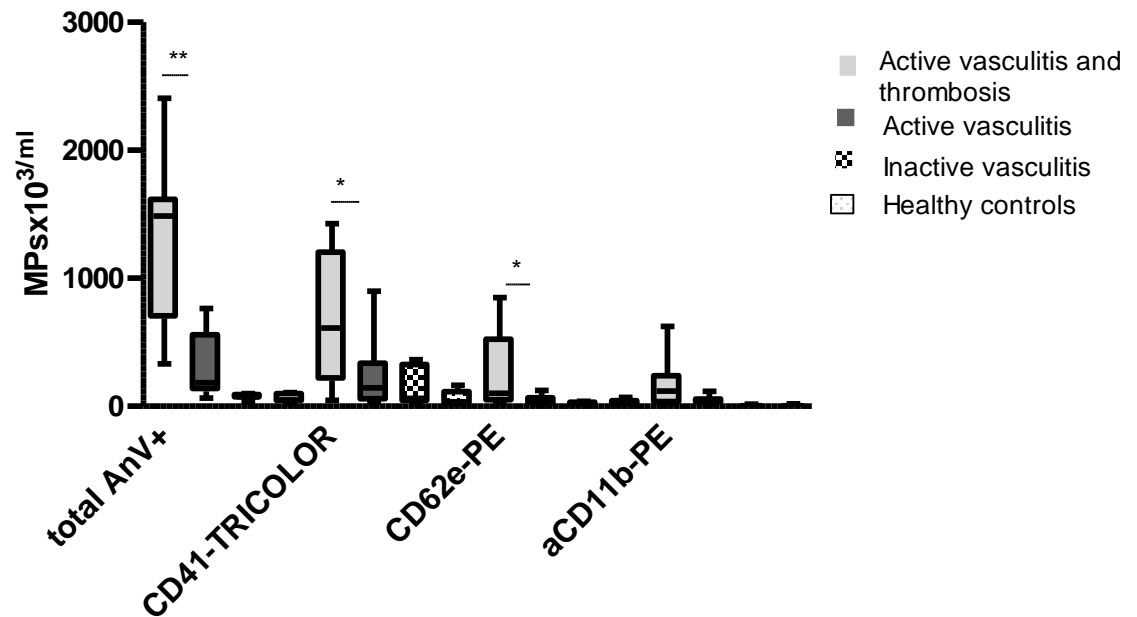
### **5.6.6 Total AnV+ MPs, PMPs and EMPs are higher in vasculitis patients with thromboses**

Patients with active vasculitis and thromboses had significantly higher total AnV+ MPs (1487 x10<sup>3</sup>/ml, range 331-2406 x10<sup>3</sup>/ml); PMPs (940 x10<sup>3</sup>/ml, range 470-1100 x10<sup>3</sup>/ml); and EMPs (455 x10<sup>3</sup>/ml, 240-749 x10<sup>3</sup>/ml) than children with active vasculitis and no thromboses (total AnV+ MP 270 x10<sup>3</sup>/ml (64-764 x10<sup>3</sup>/ml), p=0.001; PMPs 234 x x10<sup>3</sup>/ml (32-300 x10<sup>3</sup>/ml), p=0.02; EMPs 23.96 x10<sup>3</sup>/ml (2-123 x10<sup>3</sup>/ml), p=0.04; (Figure 5-10).

There was no significant difference in NMPs between these patient groups, p=0.514.

Circulating TF+ MPs were found at lower levels than PMP or EMP in all patient groups. There was no significant difference in TF+ MPs in children with active vasculitis and thromboses (median 7 x10<sup>3</sup>/ml, range 1.066-25.051/ x10<sup>3</sup>/ml) compared to those patients

with active vasculitis without thromboses (median 3.350  $\times 10^3$ /ml, range 2.1-3.8  $\times 10^3$ /ml,  $p=0.4$ ); or those with inactive vasculitis (median 2.5  $\times 10^3$ /ml, range 2-3.6  $\times 10^3$ /ml,  $p=0.14$ ) and healthy controls median 2.15  $\times 10^3$ /ml (range 0.1 -6.7 /ml  $\times 10^3$ /ml),  $p=0.05$ ).



**Figure 5-10: Circulating microparticle (MP) profiles in children with systemic vasculitis and thrombosis.**

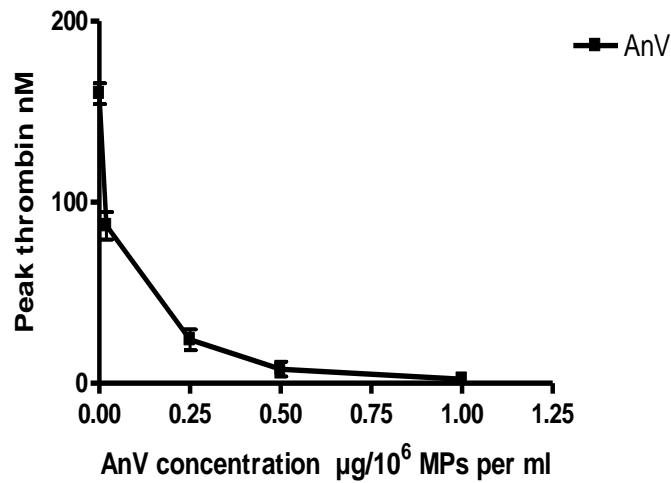
*Platelet MPs were defined as CD41-TRICOLOR+AnV+; neutrophil derived MP as aCD11b+AnV+; and endothelial derived MP as CD62e+Anv+. \*\*P < 0.001 with Mann Whitney U test.*

### ***5.6.7 Relative contribution of PS and TF to MP mediated thrombin generation***

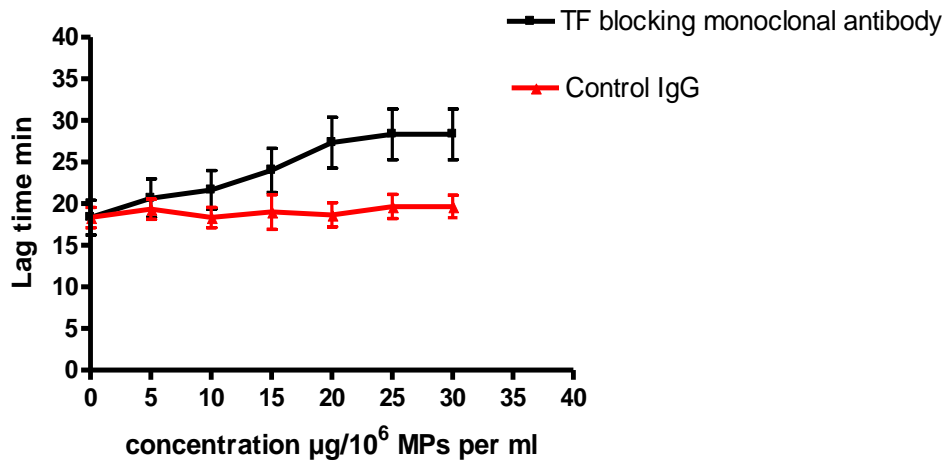
Increasing amounts of AnV protein (to bind and inhibit PS) completely abrogated thrombin generation mediated by MPs derived from LPS stimulated whole blood: thrombin generation was reduced by 99% by 1  $\mu\text{g}$  of AnV / $10^6$  MPs (figure 5-11). These results suggest that the procoagulant properties of MPs are largely due to the PS exposure.

TF neutralisation (maximum inhibition established from previous experiments at a concentration of 20  $\mu\text{g}$ /  $10^6$  MPs- see methods section) had the most significant effect on the initial phase of thrombin generation resulting in prolongation to control values of lag time 28 (24–30) min, whilst a purified IgG1 negative control blocking antibody had no effect (figure 5-12).

Incubation of MP suspensions obtained from patient samples with AnV at 1  $\mu\text{g}/10^6$  MPs again completely abolished thrombin generation. The effect of TF neutralization on MP mediated thrombin generation for children with vasculitis also was similar to that observed using MP generated using LPS from whole blood with a prolongation of lag time to 29 (range 23-32 mins).



*Figure 5-11: Increasing concentrations of Annexin V abolished microparticle mediated thrombin generation.*



*Figure 5-12: Tissue factor inhibition of microparticle mediated thrombin generation. An anti-human tissue factor antibody resulted in an average prolongation of lag time by 10 min (8–12) to control values of median 28 (24–20) min whilst a purified IgG1 negative control blocking antibody had no effect.*

### ***5.6.8 Differential contributions of monocyte and platelet-derived microparticles towards thrombin generation***

Platelet and monocyte MPs resuspended in MPFP at a concentration of  $10^6$  /ml were compared as for their ability to initiate thrombin generation.

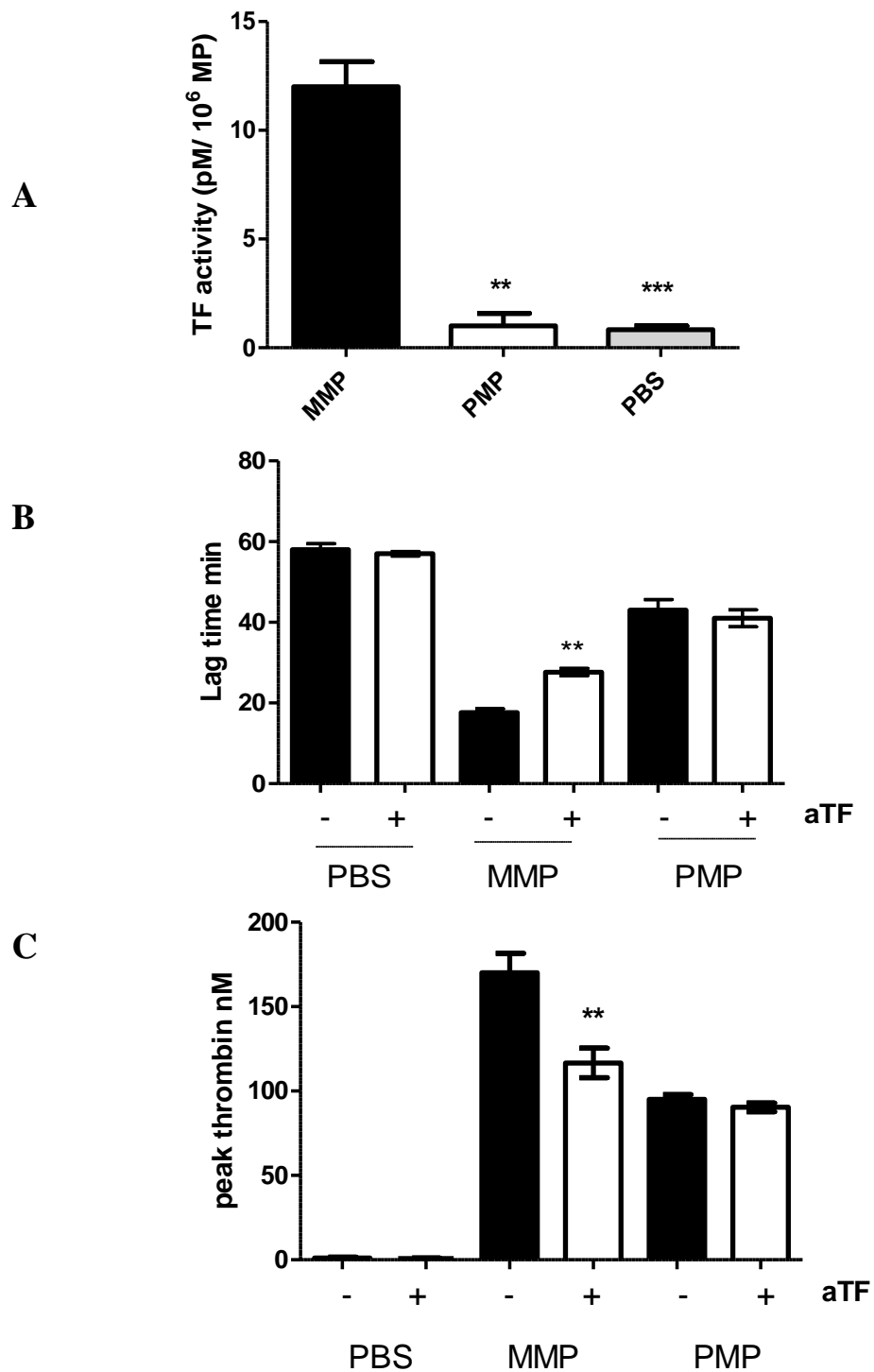
Figure 5-13 A shows TF activity on monocyte derived MPs ( $12 \pm 2$  pM TF per  $10^6$  MP). However, PMPs, regardless of the agonist used for their derivation, did not generate FXa ( $1 \pm 0.6$  pM TF per  $10^6$  MP) suggesting PMPs do not support tissue factor pro-coagulant activity.

PMPs shortened the lag time  $43.00 \pm 2.646$  min compared to  $58.00 \pm 1.528$  min for PBS ( $p=0.008$ ). PMPs also increased the peak thrombin  $95.00 \pm 2.887$  nM over PBS controls  $1.000 \pm 0.5774$  nM,  $p=0.001$  (figure 5-13 B and C). These activities were not blocked by anti-TF antibody (concentration  $20 \mu\text{g}/10^6\text{MP}$ ) peak thrombin  $95.00 \pm 2.887$  nM at baseline and  $90.33 \pm 2.603$  nM after TF neutralisation indicating this activity was TF independent.

In contrast monocyte MPs significantly shortened the thrombin lag time  $17.67 \pm 0.8819$  min compared to  $58.00 \pm 1.528$  min for PBS ( $p=0.0001$ ) and increased the peak thrombin nM  $170.0 \pm 11.55$  nM compared to  $1.000 \pm 0.5774$  nM in control PBS ( $p=0.001$ ) in a TF-dependent manner (peak thrombin  $116.7 \pm 8.819$  nM,  $p=0.003$  following TF neutralisation).

These findings suggest MPs from different parent cells uniquely contribute to coagulation, and that the relative concentration of circulating MPs from different cell types influences thrombosis risk.





**Figure 5-13: Monocyte-derived microparticles (MMPs), but not platelet-derived MPs (PMPs), promote thrombin generation in a tissue factor (TF)-dependent manner.**

(A) TF activity was determined by factor (F) Xa chromogenic substrate cleavage ( $n = 3$ ). (B) and (C) Thrombin generation supported by  $10^6$  PMP or MMP was measured in the presence of anti-TF or control IgG. Thrombin lag time and peak thrombin nM are shown. PBS=phosphate buffer saline. \*\* $P < 0.001$  using paired student's *t*-test between anti-TF and IgG controls.

## 5.7 Discussion

The mechanisms contributing to thrombosis in systemic vasculitis are poorly defined and the increased risk is not explained by conventional pro-thrombotic risk factors (Sebastian et al. 2007; Tomasson, Monach, & Merkel 2009). This study demonstrates for the first time enhanced MP-mediated thrombin generation in children with active vasculitis, with significantly higher levels of thrombin generation in those with documented thrombotic events. Thrombin generation correlated significantly with the total number of plasma MPs, suggesting that the hyper-coagulability in vasculitis is directly related to the number of these circulating MPs. These data suggest that MP mediated thrombin generation provides a novel prothrombotic mechanism, which, in the context of endothelial injury and structural arterial damage (such as aneurysm formation and/or arterial stenoses), could explain the pathological thrombosis observed in vasculitis.

Thrombo-embolic disease complicating systemic vasculitis is associated with significant morbidity and mortality (Tomasson, Monach, & Merkel 2009). Most VTEs occurred during or closely following periods of active disease (Merkel et al. 2005; Tomasson, Monach, & Merkel 2009; Weidner et al. 2006). Subsequently Sebastian et al. found no association between the presence of conventional prothrombotic risk factors and VTE in the same cohort of patients (Sebastian et al. 2007). This current study also now confirms lack of contribution of conventional prothrombotic risk factors to the increased thrombosis observed in children with vasculitis. Vasculitis could increase thrombotic risk via several mechanisms including: endothelial injury and TF exposure on endothelial cells and monocyte; platelet activation;

structural arterial injury including aneurysm formation and /or stenoses resulting in altered shear stress forces and flow dynamics; anti plasminogen antibodies compromising fibrinolysis or other as yet undefined mechanisms (Berden et al. 2010; Brogan et al. 2009; Trifiletti, Scamardi, Bagnato, & Gaudio 2009). Our lack of clear understanding of the relative contribution of each of these possibilities makes primary thrombosis prevention strategies challenging, with lack of suitable biomarkers that could define risk in routine clinical practice.

Previous studies have shown that it is possible to monitor endothelial injury in systemic vasculitis in children by the detection of circulating EMPs allowing non-invasive tracking of vasculitic disease activity, an observation now also confirmed in adults with ANCA associated vasculitides (Brogan et al. 2004; Clarke et al. 2010; Erdbruegger et al. 2008). The majority of these studies of circulating MPs in systemic vasculitis have been based on flow cytometric quantification of numbers of MPs in circulation rather than study of their functional properties (Brogan et al. 2004; Clarke et al. 2010; Erdbruegger et al. 2008). A modified thrombin generation assay was employed herein to explore for the first time the prothrombotic properties of MPs in children with systemic vasculitis. This is an important pathway to study in this context since the generation of thrombin is essential in haemostasis and thrombosis. Produced at sites of vascular injury in the vicinity of a thrombus, thrombin promotes clotting and clot stability by modulating fibrin properties, including its network structure and resistance to fibrinolysis (Wolberg 2007). Thrombin in addition promotes platelet activation and adhesion, and trafficking of inflammatory cells into sites of vascular injury (Davey et al. 1967; Strukova 2001). In addition, the important role of thrombin as a

key molecule in relation to venous thrombotic disease is largely evident from medical practice where treatment and prevention of venous thrombosis require medication that diminish thrombin activity by either inhibiting prothrombin synthesis (vitamin K antagonists) increasing antithrombin activity (heparins), or by inhibiting thrombin directly (hirudin and small-molecular-weight reversible inhibitors) (van Geffen & van Heerde 2012). Automation of the thrombin generation assay by Hemker et al. has led to an increased use of the technique to appreciate hypercoagulable states in several conditions associated with increased thrombotic risk while the current methodology used is reproducible and well suited for clinical applications (Hemker, Willems, & Beguin 1986; Hemke et al.1993; Hemker et al. 2003; Hemker & Beguin 1995). Of note, a number of previous studies have suggested that contact factor activation may influence thrombin generation results (Hemker, Wielders, Kessels, & Beguin 1993; Luddington and Baglin 2004). Thus corn trypsin inhibitor (CTI) to inhibit contact pathway activation may be useful in measurements of plasma thrombin generating capacity particularly in those with low thrombin generating potential and was therefore used herein. In addition, no exogenous TF to initiate coagulation was added to the samples to allow for measurements of the TF present in patient MP.

The present study demonstrates that MP-mediated thrombin generation is significantly elevated in children with active systemic vasculitis compared with those with inactive disease and healthy controls, and in patients with thrombosis compared with those without and significantly higher counts of total circulating MP as well as EMP, and PMP. Indeed, the number of circulating annexin V+MP (PS rich) correlated with peak thrombin generation suggesting that the MP hyper-coagulability is a direct consequence of their PS content. The

thrombin generating capacity of these MPs was almost completely abolished (99% reduction) after incubation with recombinant AnV, confirming that PS exposure is the major determinant of MP related coagulation in this context. Neutralisation with pure TF indicated that increasing TF shortens the TGA lag time in a dose-dependent manner to healthy control values but had a lesser effect on peak height. TF bearing MP (monocyte derived MPs) independently initiated thrombin generation while PMPs supported plasma thrombin generation in a TF independent manner. These findings suggest different roles of these MPs in haemostasis and thrombosis. MPs derived from different parent cells appear to uniquely contribute to TF-dependent and -independent activities that promote thrombus formation and growth, a finding replicated in recent studies (Aleman et al. 2011;van der Meijden et al. 2012).

The observations made in this thesis could have important clinical and therapeutic implications. ROC analysis of peak thrombin as a diagnostic test for thrombosis in systemic vasculitis was significant and suggests that the MP mediated thrombin generation assay could be useful in identifying children with vasculitis at risk of thrombosis. TGA in clotting blood or plasma can be used to assess the overall function of the coagulation cascade and define a unique coagulation phenotype for individual patients thus enabling risk stratification for thrombosis prophylaxis. Moreover, enhanced MP thrombin generation as a mechanism underlying thrombotic complications in systemic vasculitis could provide a rationale for the therapeutic use of oral direct thrombin inhibitors that are receiving attention as safe and effective alternatives to cumbersome anti-coagulation with warfarin (Di Nisio et al. 2005; Gustafsson 2003). Lastly, filtration through 0.2 $\mu$ m pore size filter (similar to the filter used

for therapeutic plasma exchange) effectively removed MP from plasma and abolished MP mediated thrombin generation. Thus removal of procoagulant MP may be a target for therapeutic plasma exchange and justify the use of this intervention in severe cases of thrombosis and vascular injury.

An important limitation of the present study is that blood was obtained for analysis after thrombotic events had occurred. Further prospective longitudinal studies of vasculitis patients are thus now warranted in order to assess whether this increase in MP mediated thrombin generation could be secondary to the thrombotic event. In addition, the study included only clinically apparent thromboses in the cohort described, thus potentially underestimating the true frequency of subclinical thrombotic events in systemic vasculitis. Furthermore, this was a heterogeneous and small patient population, since systemic vasculitis is rare in children. Studying paediatric patients has important advantages, however, since this young cohort was free of other co-morbid conditions such as atherosclerosis that could influence the risk of thrombosis via increased MP production (Chironi et al. 2006; Tan and Lip 2005). Moreover, the *in vitro* capacity of plasma to generate thrombin over time must be distinguished from *in vivo* markers of thrombin generation as an ongoing clotting process in the body such as prothrombin fragments 1 and 2 (F1+2), thrombin anti-thrombin complexes and fibrinopeptide A (Teitel et al. 1982). This increased or decreased *in vitro* thrombin generation means that the function of the coagulation process is abnormal, does not necessarily mean ongoing pathology but indicates an increased risk of thrombosis or bleeding. Furthermore, MP function was analyzed from isolated parent cells studied *in vitro*; MP produced *in vivo* may possess unique properties and

therefore it will be important to compare the contributions of MP from different parent cells in *in vivo* thrombosis models. Lastly, during the thrombin-generation assay, part of the thrombin formed binds to  $\alpha$ 2-macroglobulin in plasma and accumulates as thrombin- $\alpha$ 2-macroglobulin complexes that remain active against the fluorogenic substrate but do not activate coagulation, leading to a stable level of apparent residual thrombin activity at the end of the reaction. Some authors suggest that this residual thrombin- $\alpha$ 2-macroglobulin activity may affect the results of the assay (Hemker, De Smedt, & Al Dieri 2007; Wagenvoord, Deinum, Elg, & Hemker 2010). Chandler et al. more recently have however shown that the correction for residual thrombin- $\alpha$ 2-macroglobulin activity had only a minor effect on peak thrombin generation, producing results that were on average 2% lower than the uncorrected values with high correlation ( $r^2 = 0.99$ ) (Chandler and Roshal 2009). Therefore in this study no correction for residual thrombin- $\alpha$ 2-macroglobulin activity was performed but it would be of considerable interest to further explore the need for this in future studies of children with systemic vasculitis and thrombosis.

In conclusion, this study supports an important role for cell-derived microparticles in thrombosis complicating systemic vasculitis of the young. MP-mediated thrombin generation could provide a novel means of assessing prothrombotic risk in patients with systemic vasculitis, allowing improved risk stratification and targeting of primary thrombosis prevention. Elevated MP-dependent thrombin generation constitutes a new type of hypercoagulable state, deserving further exploration in other vascular disorders where thromboembolic phenomena prevail such as childhood AIS recurrence. The role of MP mediated thrombin generation in childhood AIS is explored in chapter 6.

# 6 Microparticle mediated thrombin generation in children with arterial ischaemic stroke

## 6.1 Summary

**Background:** Thrombosis is implicated in the pathogenesis of childhood arterial ischaemic stroke (AIS). Notably however the majority of children with childhood AIS have no identifiable traditional genetic or acquired thrombophilias to account for an increased thrombotic propensity. In chapter 4, children with recurrent AIS were demonstrated to have increased circulating MPs of endothelial, platelet and neutrophil origin compared to children with a single event. These cellular MPs are prothrombotic and may contribute to hypercoagulable states associated with AIS recurrence. In addition previous studies have suggested that Von Willebrand Factor (vWF) antigen levels provide evidence of endothelial dysfunction relating to cerebrovascular inflammation and thrombosis.

**Objective:** To assess the procoagulant capacity of circulating MP in children with recurrent AIS compared to those with a monophasic disease course. Levels of vWF were also evaluated in the two study groups.

**Methods:** Single centre cross-sectional study of 46 children with AIS and cerebral arteriopathy matched with 20 paediatric controls. AIS recurrence was defined as new acute neurological deficit with radiological evidence of further cerebral infarction. Patients were considered in two groups based on AIS recurrence. MP mediated thrombin generation was



assessed using a fluorogenic assay. vWF antigen levels were assessed using a commercially available ELISA.

**Results:** Forty six children with AIS aged 8.4 (0.9-17.4) years and 20 paediatric controls aged 9 (1.2-16) years were included. Ten children had AIS recurrence while 36 had a single AIS event. Children with AIS recurrence had significantly enhanced MP-mediated peak thrombin generation compared to children with a single event,  $p=0.0001$  and healthy control children,  $p=0.0020$ . MP mediated endogenous thrombin potential (ETP), lag time and velocity index were also significantly different between patient groups. Levels of plasma vWF antigen did not significantly differ between children with AIS recurrence and children with a single event,  $p=0.8700$  but were higher compared to controls,  $p=0.0100$ .

**Conclusion:** Enhanced MP-mediated thrombin generation was demonstrated in children with recurrent AIS. MP-mediated thrombin generation combined with endothelial injury and platelet activation contributes to the thrombotic events associated with childhood AIS recurrence.

## 6.2 Introduction

Thrombosis, the result of a complex interplay between platelet activation and activation of the coagulation cascade, is a key pathophysiological mechanism in many cardiovascular and cerebrovascular disorders, including childhood AIS (Fuster et al. 1988; Heemskerk et al. 2002). Blood flow through the affected cerebral arteries may be reduced or absent, which results in focal ischaemia (Hinton 1998; Powers 1991). Because of poor collateral blood flow in the brain, infarction then occurs (Hinton 1998; Powers 1991). Under physiological

conditions, the haemostatic process controls haemorrhage after injury but at the same time, if overactive, may have the potential to precipitate diseases such as thrombosis associated with AIS (Dahlbäck 2000; Haywood, Liesner, Pindora, & Ganesan 2005). Inherited thrombophilia risk factors, particularly antithrombin, protein C, and protein S deficiency and elevated lipoprotein (a), have been found in small case series and case-control studies to be associated with AIS in children (Kenet et al. 2000; Kenet et al. 2010; Nowak-Gottl et al. 1999). Furthermore, a recent metanalysis indicated that several genetic prothrombotic conditions appear to contribute to the risk of childhood AIS (Kenet et al. 2010). As noted by Sträter et al. it is likely however that in many instances thrombophilia is a permissive factor rather than an isolated cause for AIS (Sträter et al. 2002b). In addition transient acquired prothrombotic risk factors particularly in the setting of systemic infection may contribute to thrombosis in childhood AIS (Bernard et al. 2010; Manco-Johnson et al. 1996; van Ommen et al. 2002). However our lack of clear understanding of the relative contribution of each of these factors to the thrombotic propensity underpinning childhood AIS recurrence makes prevention strategies challenging, with lack of suitable biomarkers that could define risk in routine clinical practice.

Alternative approaches of quantifying the risk of thrombosis in relation to the composite effect of candidate thrombophilias, regardless of whether they are known or unknown and regardless of whether they are genetic or acquired are now emerging (van Geffen & van Heerde 2012; Van Veen, Gatt, & Makris 2008). Determination of the thrombin-generating potential as an alternative 'global testing' strategy has been validated in that context (van Geffen & van Heerde 2012; Van Veen, Gatt, & Makris 2008). A number of previous studies

have shown that thrombin generation capacity predicts likelihood of thrombosis recurrence with hazard ratios from 2.5 to 4.0 (van Geffen & van Heerde 2012; Van Veen, Gatt, & Makris 2008). Of note, Carcaillon et al. have recently reported a significant association between thrombin generation and the risk of AIS in adults and therefore suggested that hypercoagulability has an important role in the pathogenesis of AIS (Carcaillon et al. 2011). This was a prospective cohort including 9294 subjects compared with a random sample of 1177 controls and follow up for 4 years (Carcaillon et al. 2011). A multivariate analysis showed that high levels of ETP and peak thrombin were associated with an increased risk of AIS (hazard ratio 1.16 with 95% CI, 0.90 to 1.50 and 1.31 with 95% CI, 1.01 to 1.69 for a 1 SD increase, respectively) thus suggesting that thrombin generation is an independent predictor of AIS (Carcaillon et al. 2011). In addition to the investigation of 'global haemostasis' through the use of thrombin generation potential there is a current emerging interest in the contribution of cellular microparticles to increased risk of thrombosis (Morel et al. 2006; Piccin, Murphy, & Smith 2007; Zwicker, Trenor III, Furie, & Furie 2011). MPs are known to play a major role in propagating thrombus formation through PS exposure, TF expression and TF delivery in the vicinity of activated platelets (Morel et al. 2006; Piccin, Murphy, & Smith 2007; Zwicker, Trenor III, Furie, & Furie 2011). In chapter 5 an efficient method to measure the contribution of circulating MP to haemostasis using an in vitro MP mediated thrombin generation assay was established. This assay may be an invaluable tool to detect MP related hyper-coagulability in vascular disorders linked with thrombosis such as in childhood AIS.

Children with recurrent AIS have been previously shown to have elevated levels of circulating MP compared to those children with a single event and controls. These prothrombotic MPs could be contributing to AIS recurrence pathogenesis. To date however no study has investigated whether the MP mediated thrombin generation may be associated with childhood AIS recurrence.

### ***6.2.1 Von Willebrand Factor (vWF) and vascular disorders***

Von Willebrand factor (vWF) is released by endothelial cells when these are damaged, and thus vWf levels have been proposed as a possible indicator of endothelial cell dysfunction (Blann and McCollum 1994; Lip and Blann 1997). vWF is also known to have an important function in platelet aggregation and adhesion (Ikeda et al. 1991). In addition, several studies have suggested that high concentrations of vWF may be an indirect indicator of thrombosis (Martinelli 2005). For instance vWF appears to be an index of increased risk for reinfarction and mortality in patients with angina and myocardial infarction (Jansson et al. 1991; Montalescot et al. 1998). Interestingly, increased vWF levels are also well reported to be associated with ischaemic cerebrovascular events in adults (Blann et al. 1999; Catto et al. 1997). The possible mechanisms for the association between vWF and cerebrovascular events in adults include endothelial dysfunction associated with cerebral thrombosis or other atherosclerosis related risk factors, such as hypertension or ischaemia induced release from infarcted tissue (Blann et al. 1993; Conlan et al. 1993). In a pilot study of 64 patients with acute stroke, Blann et al. we have recently demonstrated significant endothelial dysfunction with high vWF levels in conjunction to raised fibrinogen levels, plasma viscosity and raised

soluble adhesion molecule P-selectin levels (Blann, et al. 1999). These abnormalities may act synergistically to contribute to the pathogenesis of acute stroke and its complications. Furthermore, Hutchinson et al. in a study reporting on the treatment and long term outcomes of children with primary angiitis of the CNS (cPACNS), demonstrated vWF as a surrogate marker of cerebrovascular injury (Hutchinson et al. 2010). The median vWF levels in the 19 children with biopsy proven cPACNS included in the study was 1.73 IU/ml (range 0.61-3.74) with 5 children having significantly raised levels  $> 1.92$  IU/ml (Hutchinson et al. 2010). Therefore vWF may be a promising soluble marker of endothelial injury also linked to thrombosis that has not been previously studied in relation to childhood AIS recurrence.

## **6.3 Aims**

The aim of this study was to compare the MP-mediated thrombin generating capacity between children with recurrent AIS and those with a single event. Levels of vWF as an additional soluble marker of endothelial injury linked to thrombosis were examined.

## **6.4 Methods**

### ***6.4.1 Patient population***

For inclusion/exclusion criteria, patient population clinical and radiological features as well as patient group classification, healthy controls and disease controls (children with cerebral AVM) see chapter 2.

### ***6.4.2 Assessment of traditional prothrombotic risk factors***

Traditional prothrombotic risk factors were assessed based on methods described in chapter 5.

### ***6.4.3 Microparticle mediated thrombin generation***

The MP mediated thrombin generation was assessed as described in chapter 5 using a fluorogenic thrombin generation assay (TGA).

### ***6.4.4 Von Willebrand factor antigen levels***

Levels of vWF antigen were assessed using a commercially available ELISA.

## **6.5 Statistical analysis**

Values are presented as median (range) unless otherwise specified. The Kruskal-Wallis test was used to examine overall differences in experimental laboratory markers between the study groups. Following this, statistical differences between groups were determined by Mann-Whitney U test. Associations between cumulative MP counts and peak thrombin generated were assessed using Spearman's rank correlation coefficient. A multivariable logistic regression was used to explore the relationship of peak thrombin to the outcome of AIS recurrence adjusted and unadjusted for age/sex and time from index AIS to evaluation. Results are expressed as OR with 95% CI. P-values of less than 0.05 (two sided) were regarded as significant. Statistical analysis was performed using SPSS version 16 and 17.

## **6.6 Results**

### ***6.6.1 Traditional thrombophilia risk factors***

With regards to traditional thrombophilia risk factors screen one child in the group with recurrence was heterozygous for methylenetetrahydrofolate reductase (MTHFR) mutations (10%) and one initially had low protein S (10%) that subsequently recovered to normal levels. In the group of children with no recurrence two patients had protein S deficiency (5%); one child was MTHFR mutation homozygous (3%) with 3 being heterozygous (8%); lastly one child was heterozygous for factor V Leiden deficiency (3%).

### ***6.6.2 Microparticle mediated thrombin generation in children with recurrent AIS***

Higher levels of AnV<sup>+</sup> MPs (confirming high pro-coagulant phosphatidylserine content) were previously shown to be present in the circulation of children with AIS recurrence compared to those children with a single event (chapter 4). Having established a reproducible method to assess the capacity of MP to generate thrombin in chapter 5, a similar approach to examine the differences in the pro-coagulant properties of circulating MP in children with AIS recurrence compared to those with a monophasic disease course was used herein. TGA parameters for all studied groups are summarized in table 6-1. Children with AIS recurrence had significantly enhanced MP-mediated peak thrombin generation 127.3 nM (30-215nM) compared to children with a single event 28 nM (10-73.40

nM),  $p=0.0001$ ; healthy control children 38.75 nM (17.70-61 nM),  $p=0.0020$ ; and children with cerebral AVM 35 nM (17-65 nM),  $p=0.0030$ . MP mediated ETP was also significantly higher in those with recurrence 2620 nM x min (800-4671nM x min) compared to those with no recurrence 1118 nM x min (451-2218 nM x min),  $p=0.0002$ ; child controls 1100 nM x min (501-1808 nM x min),  $p=0.0030$ ; and children with cerebral AVM 1200 nM x min (600-1900 nM x min);  $p=0.0040$ . Children with recurrent AIS had significantly shortened lag time 18 min (12-22 min) compared to those with non-recurrent AIS 26 min (22-31 min),  $p=0.0100$ ; child controls 32 min (28-36 min),  $p=0.0010$ ; and children with cerebral AVM 31 min (25-38 min),  $p=0.0020$ . Lastly patients with recurrence showed an increased velocity index 15.40 nM/min (5.7-21 nM/min) for thrombin generation compared to those with no recurrence 3.2 nM/min (2-8.6 nM/min),  $p=0.0010$ ; healthy child controls 1.5 nM/min (0.6-2.25 nM/min),  $p=0.0040$ ; and children with cerebral AVM 2.5 nM/min (0.8-3.2 nM/min);  $p=0.0040$ .

### ***6.6.3 Correlation of circulating microparticles to thrombin generation***

The total circulating AnV+ MPs correlated significantly with MP-mediated peak thrombin nM,  $r_s=0.73$ ,  $p<0.0001$  consistent with the hypothesis that the hypercoagulability in patients with AIS was directly related to the increased total number of PS expressing (AnV+) MPs (figure 6-1).



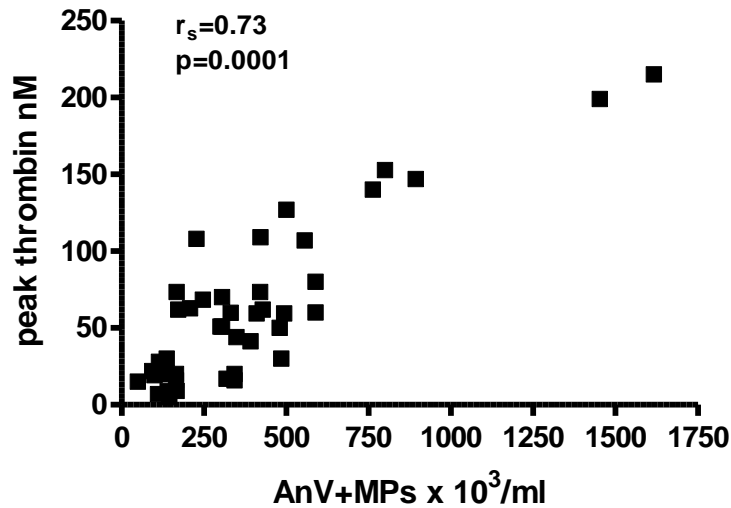
#### ***6.6.4 Von Willebrand factor (vWF) antigen levels***

Levels of plasma vWF antigen were not significantly different between children with AIS recurrence 0.850 IU/ml (0.330-2.250 IU/ml) and children with a single event 0.743 IU/ml (0.370-1.890IU/ml),  $p=0.8700$ . Levels of vWF were however significantly higher in the group of children with AIS recurrence compared to controls 0.34 IU/ml (0.31-0.7IU/ml),  $p=0.0100$  and children with AVM 0.51 IU/ml (0.4-0.632 IU/ml),  $p=0.040$  (table 6-1).

	<b>AIS recurrence (n=10)</b>	<b>AIS no recurrence (n=36)</b>	<b>Healthy controls (n=10)</b>	<b>Cerebral AVM (n=10)</b>
Peak thrombin nM Median(range)	127.3 (30-215)	28 (10-73.40); <b>p=0.0001</b>	38.75 (17.70-61); <b>p=0.0020</b>	35 (17-65); <b>p=0.0030</b>
ETP nM x min Median(range)	2620 (800-4671)	1118 (451-2218); <b>p=0.0002</b>	1100 (501-1808); <b>p=0.0030</b>	1200 (600-1900); <b>p=0.0040</b>
Lag time min Median (range)	18 (12-22)	26 (22-31); <b>p=0.0100</b>	32 (28-36); <b>p=0.0010</b>	31 (25-38); <b>p=0.0020</b>
Velocity index nM/ min Median(range)	15.40 (5.7-21)	3.2 (2-8.6); <b>p=0.0010</b>	1.5 (0.6-2.25); <b>p=0.0040</b>	2.5 (0.8-3.2); <b>p=0.0040</b>
vWF antigen IU/ml Median(range)	0.85(0.33-2.25)	0.743 (0.370-1.890); <b>p=0.8700</b>	0.34 (0.31-0.7); <b>p=0.0100</b>	0.51 (0.4-0.632); <b>p=0.040</b>

**Table 6-1: Thrombin generation parameters and von Willebrand Factor (vWF) antigen in children with arterial ischaemic stroke (AIS).**

vWF= von Willebrand Factor; AVM= arteriovenous malformation. ETP=endogenous thrombin potential (corresponding to the area under the curve for thrombin generation assay curves). The Kruskal-Wallis test followed by the Mann-Whitney U test was used to examine differences between the study groups. P values reported refer to comparison with the group of children with recurrent AIS. P-values of less than 0.05 (two sided) were regarded as significant.



*Figure 6-1: Total circulating Annexin V microparticles correlated with peak thrombin generation in children with arterial ischaemic stroke (AIS),  $r_s=0.73$ ,  $p=0.0001$ .*

### **6.6.5 Logistic regression analysis of peak thrombin and AIS recurrence**

Logistic regression analysis was used to examine the relationship of peak thrombin to AIS recurrence unadjusted and adjusted for age/sex and time from index AIS to evaluation as these could be important confounding factors (table 6-2). AIS recurrence was associated with elevated peak thrombin in both non-adjusted OR 1.515 (95% CI 1.173-1.705),  $p=0.003$  and adjusted analysis OR 1.319 (95% CI 1.040-1.429),  $p= 0.010$ .

Biomarker	Unadjusted			Adjusted (age/gender/time from AIS to evaluation)		
	OR	95%CI	P	OR	95%CI	P
Peak thrombin nM	1.515	1.173-1.705	0.003	1.319	1.040-1.429	0.010

*Table 6-2: Unadjusted and adjusted odds ratios by multivariable logistic regression analysis for arterial ischaemic stroke recurrence (AIS).*

*Adjusted odds ratios for age/gender/time from AIS to evaluation; OR=odds ratio; CI=confidence intervals. P<0.05 was considered significant.*

## 6.7 Discussion

Children with recurrent AIS demonstrated enhanced MP-mediated thrombin generation compared to children with a single event, children with cerebral AVM and child controls. MP mediated thrombin generation provides a novel mechanism of hypercoagulability that may be implicated in the pathogenesis of childhood AIS. Prospective validation of these findings is now needed to establish the prognostic value of MP mediated thrombin generation in predicting recurrence of childhood AIS. Of note, vWF antigen levels did not effectively differentiate between patient groups but were significantly raised in children with recurrence compared to controls.

Thrombus generation is one of the main causes for brain ischaemia in childhood onset AIS, whether the clot originates in the heart, the cervical arteries or the intracranial arteries (Hinton 1998). As thrombotic/embolic phenomena prevail in the pathophysiology of

childhood-onset AIS, investigation of the contribution of thrombophilias to risk of incident and recurrent AIS in childhood is critical. Although multiple types of genetic and acquired thrombophilia are established as independent risk factors for incident AIS, data supporting significant prognostic impact upon recurrence risk are limited to a few individual traits such as elevated lipoprotein (a), protein C deficiency, and the presence of multiple risk factors (Kenet et al. 2000; Kenet et al. 2010; Nowak-Gottl et al. 1999). In addition the majority of children with AIS have no identifiable traditional thrombophilia factors to account for the thrombotic/embolic phenomena associated with childhood AIS, a finding supported by this study too. As a result identifying prothrombotic tendencies relating to re-infarction remained to date a challenge. Thrombin generation, a reliable functional test of the haemostatic–thrombotic system, could now provide an effective way of evaluating the relative contribution of hypercoagulability to childhood AIS recurrence.

Enhanced MP mediated thrombin generation was demonstrated in children with recurrent AIS. This observation not only sheds light on the pathogenesis of childhood AIS recurrence but could also have several therapeutic implications. Provided that these results are confirmed in larger studies, prevention of childhood AIS recurrence should perhaps target the diminution of the thromboembolic risk together with prevention of endothelial dysfunction. In addition, novel oral anti-thrombin agents have been receiving attention as safe and effective alternatives to traditional anticoagulants (Gustafsson 2003). If excessive thrombin generation underpins childhood AIS recurrence these agents may be beneficial in secondary stroke prevention strategies. Monitoring of MP-mediated thrombin generation

may also allow risk-stratified approaches to antithrombotic therapy in order to optimise the balance between bleeding and recurrence risks.

The finding that plasma levels of vWF did not differentiate between patient groups was unexpected. The small size of the study could have influenced the results. Another possible explanation is that interpretation of raised vWF levels may be complicated by its behaviour as an acute phase reactant. Elevated plasma vWF levels have been previously associated with the risk of vascular events, an association that may be explained by a reactive or secondary rise in plasma of vWF mainly during the acute phase of inflammation (Pottinger et al. 1989). In this study, children with recurrent AIS were not studied during the acute phase of stroke and this may have affected the results. Nevertheless, measurement of vWF levels may be more useful than that of acute phase reactants such as C-reactive protein or erythrocyte sedimentation rate (ESR) as vWF implies endothelial injury (Cellucci et al. 2009; Cellucci, Tyrrell, Sheikh, & Benseler 2011; Cellucci, Tyrrell, Pullenayegum, & Benseler 2012; Cellucci & Benseler 2010). Lastly, treatment with aspirin is well established to decrease levels of vWF antigen (Pernerstorfer et al. 2001). All children with AIS studied herein were on aspirin at the time of recruitment and hence this could provide an additional explanation for lack of significant differences in vWF antigen levels between patient groups.

Limitations of this study include the small size and heterogeneity of the population studied as childhood AIS is rare. In addition whether this increased hyper-coagulability is an epiphenomenon associated with established thrombosis or has a causal effect to AIS recurrence was not determined. It remains to be established in prospective cohort studies

how testing of an expanded genetic repertoire and global haemostasis testing using the MP-mediated thrombin generation will influence clinical decision-making in relation to long-term anticoagulant therapy after index AIS. Whether this in vitro hypercoagulability is truly representative of what occurs in vivo remains unclear. However, the hypothesis that it may have clinical consequences is attractive and deserves further attention.

In summary, these observations regarding MP mediated thrombin generation and childhood AIS recurrence constitute a new and potentially clinically important finding requiring validation in larger prospective populations. MP mediated thrombin generation provides a novel link between inflammation, endothelial injury and thrombosis that provides an exciting insight into the pathogenesis of childhood AIS. Novel antithrombotic strategies may in that context prove to be more effective in select cases.

# 7 Endothelial repair in children with arterial ischaemic stroke and cerebral arteriopathy

## 7.1 Summary

**Background/aims:** An emerging paradigm relating to vascular health is the concept of the balance between endothelial injury and repair. Endothelial progenitor cells (EPC) constitute a circulating pool of cells that counteract ongoing risk factor induced vascular injury and facilitate the replacement of dysfunctional endothelium. It is now becoming increasingly apparent that several vascular disorders can unfavourably alter this balance of endothelial injury and repair resulting in significant morbidity and mortality. To date, however, virtually nothing is known about endothelial repair responses in childhood arterial ischaemic stroke (AIS).

**Methods:** Children with AIS were considered in two groups based on recurrence, and compared with healthy controls. Circulating EPC were identified with flow cytometry as peripheral blood mononuclear cells (PBMCs) triple positive for: CD34/CD133/CD144; or CD34/CD133/VEGFR2. EPC function was evaluated by the potential of these cells to form colony forming units (CFU), and by their ability to incorporate into endothelial cell vascular structures in matrigel.

**Results:** Thirty five children, median age 12 years (5–16.5 years; 9 males) with AIS (10 with recurrence) were studied. Circulating EPC triple positive for CD34/CD133/CD144 were significantly raised in children with AIS recurrence: 0.07% of PBMC gated (range 0.04-0.2%) compared to 0.02% (0-0.09%) in patients with no recurrence,  $p = 0.0001$ ; and



0.03% (range 0-0.07%) in 20 child controls,  $p=0.0001$ . Similar differences were detected in PBMCs triple positive for CD34/CD133/VEGFR2 between the study groups. EPC- CFU were significantly reduced in children with AIS recurrence compared to those with no recurrence,  $p=0.04$ ; and child controls,  $p=0.03$ . Compared with healthy controls, the number of EPC incorporated into the tubular network formed by HUVEC was reduced in children with AIS recurrence,  $p=0.02$ .

**Conclusions:** Children with recurrent AIS had evidence of increased circulating CD34+/CD133+/KDR+ cells representing an attempt at endothelial repair in response to more severe neurological injury in these children. The ability of EPCs however to grow in culture, form colonies and incorporate into endothelial cell vascular networks was impaired in children with AIS recurrence. These data suggest that there could be impairment of a potentially important pathway for endothelial repair in these patients, leading to an unfavorable balance between endothelial injury and repair. Further prospective studies with larger patient numbers are required to confirm this definitively.

## **7.2 Introduction**

Neo/revascularization is a process to restore or regenerate the damaged blood vessels in the body (Freedman and Isner 2002;Lapergue et al. 2007). Revascularization involves: vasculogenesis that refers to the de novo formation of blood vessels (Freedman & Isner 2002; Lapergue, Mohammad, & Shuaib 2007); and angiogenesis which involves the formation of new capillaries from the pre-existing vasculature, and is controlled by a number of growth factors and signalling pathways (Freedman & Isner 2002; Lapergue, Mohammad,

& Shuaib 2007). Thus, it is largely accepted that vasculogenesis may play a key role in embryogenesis, whereas angiogenesis occurs during both prenatal and postnatal life (Freedman & Isner 2002; Lapergue, Mohammad, & Shuaib 2007). Since the pioneer work by Asahara et al. however increasing evidence suggests that circulating adult bone marrow-derived endothelial progenitor cells (EPC) play an important role in neo/re-vascularization under certain physiological and pathological conditions in adults through vasculogenesis (Asahara et al. 1997). In addition, a third process, arteriogenesis, completes the expansion and growth of the vascular system in adults since it refers to the development and growth of pre-existing arterioles into physiological relevant arteries that form collateral vessels (Freedman & Isner 2002; Lapergue, Mohammad, & Shuaib 2007).

### ***7.2.1 Endothelial progenitor cells (EPC)***

Under physiological conditions the endothelium is primarily quiescent with very low rates of replication (Hirschi, Ingram, & Yoder 2008; Yoder et al. 2007). However, in response to several factors such as inflammation, hypoxia, and oxidative stress causing endothelial activation and vascular injury, multiple circulating blood cells are recruited to the area of injury, with an additional but more delayed recruitment of resident endothelium, to re-establish vascular integrity and blood flow (Haudenschild and Studer 1971; Haudenschild and Schwartz 1979; Richardson & Yoder 2011). The majority of endothelial repair probably results from the proliferation of neighbouring endothelial cells, although circulating cells are now also known to modulate vascular repair (Asahara et al. 1997; Asahara et al. 1999a; Hill et al. 2003; Richardson & Yoder 2011). In 1997 bone marrow derived progenitor cells,

termed endothelial progenitor cells (EPCs), were described that displayed a variety of endothelial specific cell surface proteins following culture in vitro; and that possessed the ability to localize and promote vascular regeneration at sites of ischaemia (Asahara et al. 1997). A wealth of studies have subsequently confirmed that these bone marrow derived EPCs incorporate into sites of neoangiogenesis and injury; however, the evidence that such cells actually become endothelial cells remains a topic of active debate (Rabelink, de Boer, & van Zonneveld 2010; Richardson & Yoder 2011). In addition, confusion has also arisen because the common term 'EPC' has been used for the description of what are now known to be completely different cellular subpopulations (table 7-1).

In general, the methods for the isolation and quantification of EPCs can be grouped into two main approaches: 1. in vitro adhesion colony formation; and 2. flow cytometric identification of circulating cells with a defined cell surface phenotype. At the time of writing this thesis there is no consensus on specific unique cell surface molecules that permit isolation of an EPC in humans. That said, it is now established that there are numerous cell types and lineages that participate in neovascularization (Rabelink, de Boer, & van Zonneveld 2010; Richardson & Yoder 2011). In addition, despite the lack of a unifying phenotypic definition for EPCs, an approach for addressing how all these different cells and cell lineages participate in the process of endothelial repair is emerging, and has provided new therapeutic strategies for enhancing (e.g. adults with peripheral vascular disease), or inhibiting (cancer medicine) the process of new blood vessel formation, depending on the clinical context (Dzau et al. 2005; Ferrara and Kerbel 2005). The following sections summarise our current knowledge and understanding of EPC biology.

### **7.2.1.1 Isolating EPC using in vitro adhesion and colony formation**

In the original paper defining EPC, Asahara et al reported that 15.7% of adult peripheral blood cells expressing CD34 could be isolated using immunomagnetic beads, and culture of this population on fibronectin-coated tissue culture wells led to the emergence of spindle-shaped cells within 3 days (Asahara et al. 1997). At 7 days of culture, the attached cells expressed a variety of endothelial cell surface proteins such as CD31, Flk-1 (vascular endothelial growth factor 2 receptor), Tie-2, or E-selectin (Asahara et al. 1997). These cultured cells also displayed the ability to take up the lectin *Ulex Europeaus* agglutinin-1 (UEA-1) and fluorescence labeled acetylated low density lipoprotein (acLDL) (Asahara et al. 1997). In addition, cells from the adherent population recovered from these cultures could home to and co-localize within capillaries of ischaemic limbs in rabbits or mice, resulting in improved blood flow (Asahara et al. 1997). Ito et al. modified the original EPC assay by allowing all mononuclear cells to attach to the fibronectin-coated dishes for 24 h before removing the non-adherent cells (Ito et al. 1999). The non-adherent fraction was replated on fibronectin coated plates, and the clusters that emerged at 7 days were named EPC colony forming units (Ito et al. 1999). Subsequently Hill et al. further modified the assay to include a 48-h pre-plating period prior to replating the non-adherent cells (Hill et al. 2003). The colonies of putative EPC that emerged from the cultured non-adherent human peripheral blood mononuclear cells were called CFU-Hill (Hill et al. 2003). It is now well established that there is an inverse relationship between the numbers circulating concentration of CFU-Hill, and risk factors for development of atherosclerosis in humans; and one study linking

low CFU-Hill with increased mortality from atherosclerosis (Hill et al. 2003). The presence of lower levels and/or impaired function of such cells has been demonstrated in different disease states, particularly cancer, arterial hypertension, diabetes, pulmonary hypertension (amongst others), and suggest that they have an important role in the maintenance of vascular health (Diller et al. 2008; Fadini et al. 2005; Hill et al. 2003; Schmidt-Lucke et al. 2005; Vasa et al. 2001; Werner et al. 2005). Recently, several groups have demonstrated that plating human peripheral blood or umbilical cord blood mononuclear cells on fibronectin-coated plates (with or without preplating steps) results in colonies that are composed of round hematopoietic cells and include myeloid progenitor cells, monocytes, and T lymphocytes (Rohde et al. 2006; Rohde et al. 2007; Yoder et al. 2007). These spindle shaped cells expressing endothelial markers do not spontaneously form blood vessels when implanted in vivo in collagen gels (i.e. do not display postnatal vasculogenic activity), but readily ingest bacteria, more in keeping with their monocyte/macrophage lineage roots (Hirschi, Ingram, & Yoder 2008; Rabelink, de Boer, & van Zonneveld 2010; Richardson & Yoder 2011; Rohde et al. 2006; Rohde et al. 2007; Yoder et al. 2007). It is now believed that these myeloid-derived EPC may be of great relevance to vascular repair through paracrine mechanisms, but are unlikely to become endothelial cells themselves (Hirschi, Ingram, & Yoder 2008; Rabelink et al. 2011). These cells are also (confusingly) referred to by different names including: pro-angiogenic haematopoietic progenitor cells, myeloid-EPC, early outgrowth EPC, or circulating angiogenic cells (amongst other names) (Richardson & Yoder 2011).

In addition to this myeloid phenotype of EPC, other investigators have identified a different type of colony of cells emerging from the plated peripheral blood mononuclear cells. Endothelial colony forming cells (ECFC), also called late outgrowth endothelial cells (OEC) or blood outgrowth endothelial cells (BOEC), typically emerge from an adult blood sample in 14–21 days (Ingram et al. 2004; Lin, Weisdorf, Solovey, & Hebbel 2000). These ECFC emerge as tightly adherent colonies with a typical cobblestone appearance and are rare in adult human blood samples with approximately 1 colony/10<sup>8</sup> mononuclear cells plated (Ingram et al. 2004; Richardson & Yoder 2011). ECFC also possess clonal proliferative potential that can be observed in single cell cultures (Richardson & Yoder 2011). ECFC have been shown to form functional vessels that connect to the host murine vessels when implanted in immunodeficient mice, thus providing evidence that they become structural cells of the endothelium in mature blood vessels (Melero-Martin et al. 2007; Yoder et al. 2007). This functional capacity of the ECFC is indicative of true postnatal vasculogenesis. Whether circulating ECFC are actively mobilized or passively sloughed off the endothelium due to shear stress remains to be determined. Impaired ECFC angiogenic tube formation is observed in some disease processes, including diabetes mellitus, and pulmonary arterial hypertension (Tan et al. 2010; Toshner et al. 2009).

Interestingly Yoon et al. demonstrated that EPC of myeloid origin and ECFC may participate synergistically in neovascularization (Yoon et al. 2005). In a mouse hind limb ischemia model, as well as in a matrigel plug model, both these types of cells individually enhanced angiogenesis, but injecting of a mixture of both populations yielded the most robust angiogenic response (Yoon et al. 2005).

### 7.2.1.2 Defining EPC based on cell surface phenotype

Another EPC phenotype comprises a bone marrow-derived subpopulation of haematopoietic stem cells which are positive for CD34 and co-express VEGFR2 (also known as KDR) as identified using flow cytometry (Hirschi, Ingram, & Yoder 2008; Rabelink, de Boer, & van Zonneveld 2010). Further analysis of the cell surface proteins expressed on this subset included identification of CD133, CD144, CXCR4, CD31, CD13, but not CD14 or CD15 (Peichev et al. 2000; Rabelink, de Boer, & van Zonneveld 2010). Isolated CD34+ EPCs and CD34+VEGFR2+ EPCs are able to incorporate into endothelial mono layers *in vitro* and may contribute to *in vivo* repair of the endothelium (Peichev et al. 2000; Pelosi et al. 2002; Rookmaaker et al. 2005). Interestingly recent studies have suggested that activated platelets, platelet products and fibrin, appear to support the migration of these CD34+ cells to the site of vascular injury (de Boer et al. 2011). Irrespective of their fate, the CD34+ EPC level may correlate with endothelial activation and dysfunction (Fadini et al. 2009; Surdacki et al. 2008).

To summarise several assays have been used over the years to determine whether a specific progenitor population has endothelial cell potential (table 7-1) (Richardson & Yoder 2011). Some phenotypic characteristics and behaviors that are often specifically attributed to endothelial cells are in fact shared by other cell types, notably myeloid cells. Currently different types of blood cells and endothelial cells are known to participate in vascular repair. The levels and impaired function of these cells as identified using flow cytometry and

a number of in vitro culture assays have been linked to cardiovascular disease burden, and poor long term outcomes.

<b>Year</b>	<b>Author</b>	<b>Cell population</b>	<b>Method Used to Isolate</b>	<b>Marker to define</b>	<b>Term</b>
1997	Asahara	CD34 or KDR peripheral blood cells	Immunomagnetic bead separation of CD34 or KDR positive adult peripheral blood cells	CD45, CD34, CD31, Flk-1, Tie-2 E-selectin, UEA-1, acLDL	EPC
2000	Peichev	CD34, AC133, and KDR peripheral blood cells	Immunomagnetic bead separation of CD34 positive adult peripheral blood cells	CXCR4, CD31, CD13, acLDL, and not CD14 or CD15	CEP
2001	Lin	CD146 (P1H12) peripheral blood cells	Plating of mononuclear cell pellet or immunomagnetic bead separation of peripheral blood cells CD146 positive adult	CD34, CD144, vWF, Flk-1, and not CD14	BOEC
2003	Hill	Mononuclear cells	48 h pre-plating period prior to replating non adherent blood mononuclear cells and colony formation 4–9 days later	CD31, Tie2, and KDR	CFU-Hill
2004	Ingram	Mononuclear cells	In vitro colony emergence and clonal proliferative potential	CD34, CD146, CD31, CD105, and not CD45 and direct demonstration of vessel formation in vivo	ECFC



2007	Rohde	Mononuclear cells	48 h pre-plating period prior to replating non-adherent blood mononuclear cells and colony formation 4–9 days later	VEGF-R1, 2, and 3, CD31, CD34, CD146, vWF, and not CD14 CD45	CFU-EC
2007	Yoder	Mononuclear cells	In vitro colony emergence and clonal proliferative potential and 48 h pre-plating period prior to replating non-adherent blood mononuclear cells and colony formation 4–9 days later	CD34, CD146, CD31, CD105, and not CD45 and direct demonstration of vessel formation in vivo	ECFC Vs. CFUHill
2009	Prokopi	Mononuclear cells	Plating of mononuclear cell pellet on fibronectin-coated dishes and at 72 h removed nonadherent cells	CD31, vWF, UEA-1, CD4, and acLDL	EPC
2010	Estes	CD34, AC133, CD45, CD31	Polychromatic flow cytometry and cell sorting	CD34, AC133, CD45, CD31	Proangiogenic haematopoietic cell

**Table 7-1: Progenitor cells participating in endothelial repair responses.**

*Adapted from Richardson et al. (Richardson & Yoder 2011). UEA-1, Ulex Europeaus agglutinin-1; acLDL, acetylated low density lipoprotein; CEP, circulating endothelial precursor; ECFC, endothelial colony forming cells; BOEC, blood outgrowth endothelial cells; vWF, von Willebrand factor; CFU-EC, colony forming unit-endothelial cell; CFU-Hill, colony forming unit-Hill.*

### ***7.2.2 Endothelial progenitor cells in cerebrovascular diseases***

In the field of cerebrovascular diseases, previous clinical studies in adults with stroke have reported reduced EPC levels and/or function (Chu et al. 2008; Yip et al. 2008). Yip et al. showed that the levels of circulating CD34+EPCs examined by flow cytometry at 48 hours after stroke in 138 consecutive adult patients were significantly higher than in at-risk control subjects ( $P<0.05$ ) (Yip et al. 2008). Additionally, EPC levels were significantly lower in patients with severe neurological impairment than in patients with less severe impairment ( $P<0.0001$ ) (Yip et al. 2008). Low circulating EPC levels were independently predictive of severe neurological impairment at 48 hours and major adverse clinical outcomes (Yip et al. 2008). Furthermore Chu et al. showed that the EPC colony forming capacity and tube formation ability in matrigel assays were significantly reduced in adult patients with acute stroke compared with patients with chronic stroke, or healthy controls (Chu et al. 2008). In a study by Sobrino et al. increased EPC colony forming units were also shown to be associated with improved neurological outcome in a cohort of 48 adults with stroke (Sobrino et al. 2008). As far as childhood cerebrovascular diseases are concerned, Kim et al. studied 28 children with moyamoya disease prior to any surgical treatment and compared their endothelial repair responses to 12 healthy childhood controls (Kim et al. 2010). They demonstrated less tube formation by CD34+CD133+KDR+EPC and increased senescent-like phenotype in children with moyamoya disease (Kim et al. 2010).

These data suggest that altered EPC number and functional capacity are associated with endothelial dysfunction in adult stroke (Chu et al. 2008; Yip et al. 2008). Several studies emphasize that EPC participate in the ongoing maintenance of endothelial integrity and that

insufficient endothelial cell repair by EPC may play a causal role in vascular disease progression. The endothelial repair responses in children with AIS have however never been explored.

### **7.3 Hypothesis and aims:**

In this chapter the hypothesis that insufficient endothelial cell repair may play a causal role in vascular disease progression in children with AIS recurrence is examined. Defective endothelial repair could contribute to endothelial dysfunction and increased likelihood of stroke recurrence and/ or progressive arteriopathy. In this chapter endothelial repair responses in children with AIS recurrence are compared to those children with a single event.

### **7.4 Methods**

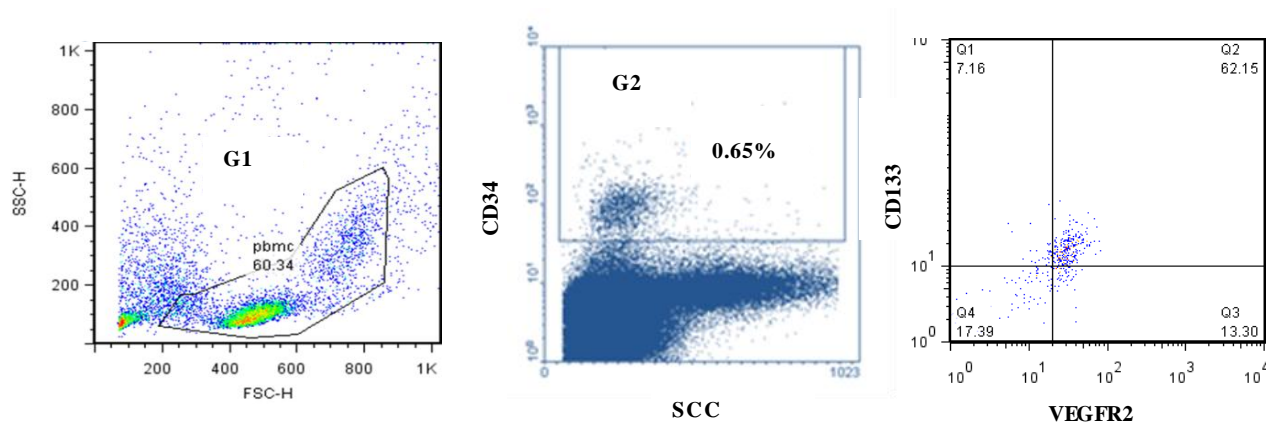
#### ***7.4.1 Fluorescent activated cell sorting (FACS) for Endothelial Progenitor Cells (EPC) surface marker identification***

To analyse PBMC for expression of cell surface protein and identify the EPC population co-expressing CD34/CD133/CD144 and CD34/CD133/VEGFR2, frozen PBMC were thawed rapidly at 37<sup>0</sup> C and re-suspended in warm FACS buffer (PRMI 1640 with 10% heat inactivated FCS) at a concentration of 2x10<sup>6</sup> cells / ml. Fifty µl of Fc receptor blocking agent

was added per ml and the cells incubated in room temperature for 10 minutes. 100  $\mu$ l of cell suspension was placed in each well of a 96-well U bottomed plate. Plates were centrifuged at 500g for 5 minutes at 4<sup>0</sup> C to obtain a cell pellet, and the solution removed by flicking. The cell pellet was re-suspended in 50  $\mu$ l of FACS buffer containing the relevant antibodies at the specified dilution (chapter 2) incubated in the dark for 30 minutes at 4<sup>0</sup> C. For VEGFR2 staining, the secondary antibody-streptavidin FITC-was added to the required wells and incubated for another 10 minutes in room temperature. After incubation, cells were washed twice with FACS buffer as above and re-suspended in 200  $\mu$ l of fixative with 10% formaldehyde with 1% azide (BD, cell FIX) and transferred to small FACS tubes for flow cytometric analysis (FACS Calibur, BD).

#### ***7.4.2 Flow cytometry data analysis***

Flow cytometric data were collected on a FACS Calibur; 20 x10<sup>3</sup> events were collected for each condition and cells gated by scatter properties. For the gating strategy used to identify the triple positive population for EPCs see figure 7-1. Non stained samples and fluorochrome matched isotype controls were used to define positivity. EPC sub-population was defined as cells co-expressing CD34 with combinations of the following endothelial and stem/progenitor cell markers: CD133, VEGFR2 and CD144. EPC numbers were expressed as a percentage of the mononuclear cell gate (figure 7-1). Data were analysed using FlowJo (Treestar Inc, Ashland, OR).



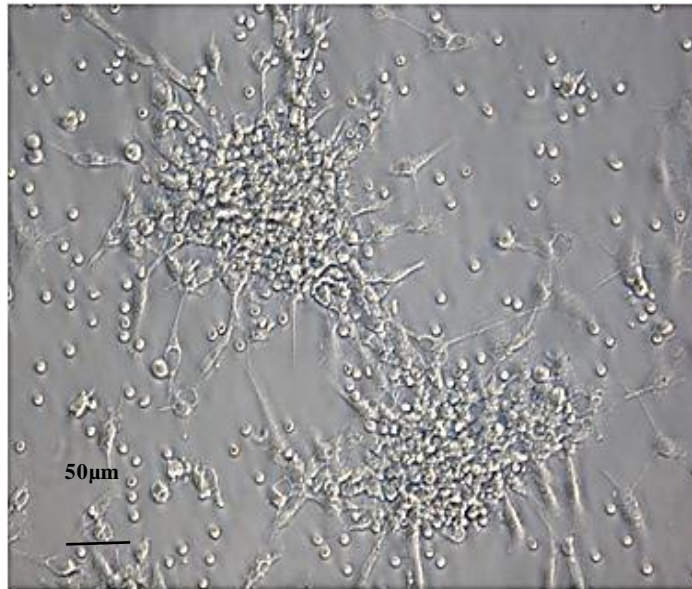
**Figure 7-1: Flow cytometry for endothelial progenitor cells (EPCs).**

Peripheral blood mononuclear cells (PBMC) were stained for VEGFR2/CD133 and CD34 and gated initially on their FSC and SSC characteristics (G1). Cells positive for CD34 were then identified (G2). G1 and G2 were combined in order to determine the proportion of VEGFR2 and CD133 positive cells. The triple positive population could then be calculated (% of PBMCs stained for VEGFR2/CD133/CD34).

### **7.4.3 Endothelial progenitor cell colony forming units (EPC-CFU)**

EPCs were further evaluated based on their in vitro ability to adhere and grow in culture giving rise to endothelial progenitor cell-colony forming unit (EPC-CFU). PBMC at  $2 \times 10^6$  isolated as described above were plated into 24 well culture plate pre-coated with human fibronectin 100ng/ml (Sigma Aldrich) and maintained in EGM-2 culture medium supplemented with growth factors as per manufacturers recommendations, 20% of FCS and 40 ng/ml of VEGF. After 4 days in culture, non-adherent cells were removed by washing

with PBS and the adherent cells maintained in culture until day 7. Early EPC-CFU were identified as a central core of rounded cells surrounded by elongated spindled-shaped cells and counted on day 8. Figure 7-2 shows a representative EPC-CFU on day 8 from EPCs isolated from PBMCS from a child with AIS at acute presentation. Cell clusters without emerging spindle cells were not counted. Colonies were counted manually in a minimum of 2 wells in 24-well plates by two independent observers who were unaware of clinical profiles; results are expressed as average number of CFUs per well. In selected samples, the endothelial phenotype was confirmed using specific indicators, that is, according to the uptake of Di-IAcLDL (Invitrogen).



**Figure 7-2: Endothelial progenitor cell- colony forming units.** Endothelial progenitor cell (EPCs) prepared from peripheral blood mononuclear cells (PBMCs) isolated from 5ml of blood from a healthy control child were plated into culture dishes coated with human fibronectin and maintained in EGM-2 supplemented with 20% fetal calf serum (FCS). After 4 days in culture, non-adherent cells were removed by washing with phosphate buffered saline (PBS) and the adherent cells maintained in culture until day 8. Early EPC colony formed units were counted in a minimum of 2 wells and expressed as average number of EPC-CFU per well.

#### **7.4.4 Human umbilical vein endothelial cell (HUVEC) capillary network formation on matrigel**

A number of studies have shown that EPCs can incorporate into vascular networks formed by endothelial cells on extracellular matrix formulations (Matrigel<sup>TM</sup>) recapitulating vasculogenesis events happening in vivo (Sieveking et al. 2008;Tepper et al. 2002). The

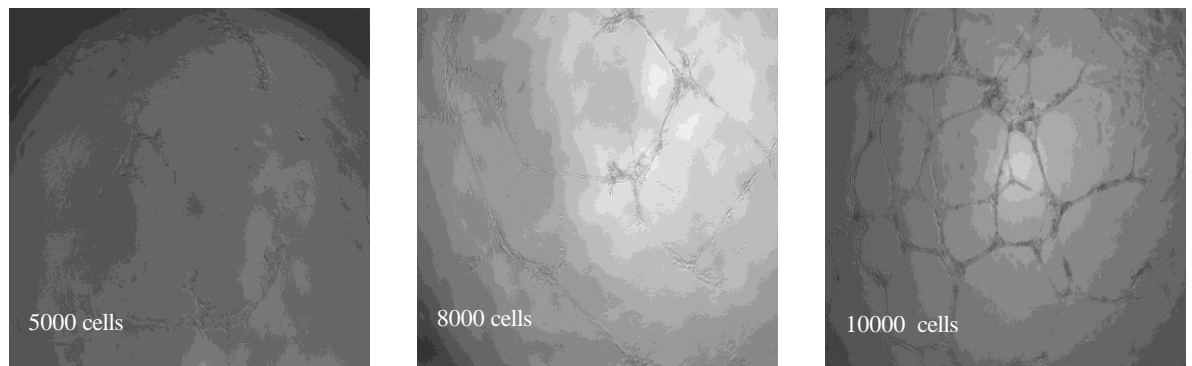
optimal number of HUVEC needed to form a capillary network in the matrigel matrix was established initially. HUVEC grown overnight in EGM-2 were treated with Trypsin/EDTA and re-suspended in EGM-2 at concentrations of 5000, 8000 and 10000 cells in 100 $\mu$ l respectively prior to being seeded onto the matrigel coated 96-well flat bottomed plate. The formation of HUVEC vascular networks (tubule formation ie a structure exhibiting length four times its width) was examined with light microscopy (figure 7-4). A concentration of 10000 per 150  $\mu$ l were subsequently chosen as the optimal concentration of HUVEC for the EPC-HUVEC incorporation assay, as at this cell concentration HUVEC exhibited maximum tubulogenesis (tubule length for 10000 cells/ 100  $\mu$ l was 8mm/mm<sup>2</sup>, range 7.6-8.6 mm/mm<sup>2</sup> versus 4.1 mm/mm<sup>2</sup>, range 3.9-4.4 mm/mm<sup>2</sup> for 8000 cells/100  $\mu$ l, p=0.02 and 2.0 mm/mm<sup>2</sup>, range 1.2-2.2 mm/mm<sup>2</sup> for 5000 cells/100  $\mu$ l, p=0.02).

#### ***7.4.5 Co-cultured endothelial progenitor cells (EPCs) and endothelial cells on matrigel***

To assess the ability of EPC to incorporate into HUVEC vascular networks the following protocol was derived: Matrigel aliquots were thawed at 4<sup>0</sup> C overnight. Fifty  $\mu$ l of the matrigel were then seeded into wells of 96-well tissue culture plates and incubated at 37C for 45 min to solidify. EPCs pre- labeled with 2 $\mu$ g/ml of Di-I-LDL for 2 hours were harvested by Trypsin/EDTA. Cells were washed 2 times with culture media (EGM-2) and then re-suspended in EGM-2 at a density of 30, 000 cells/ml. Amplified EPCs harvested and

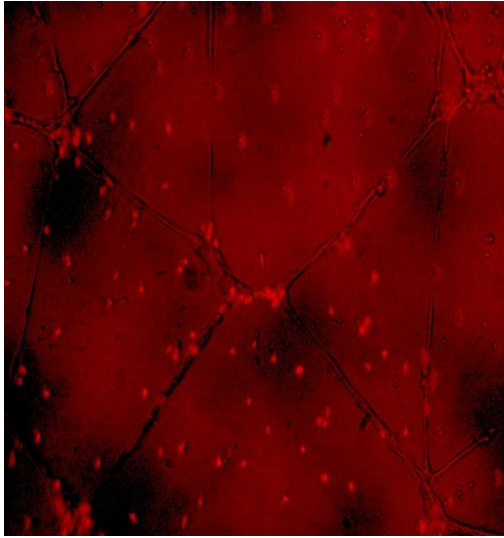
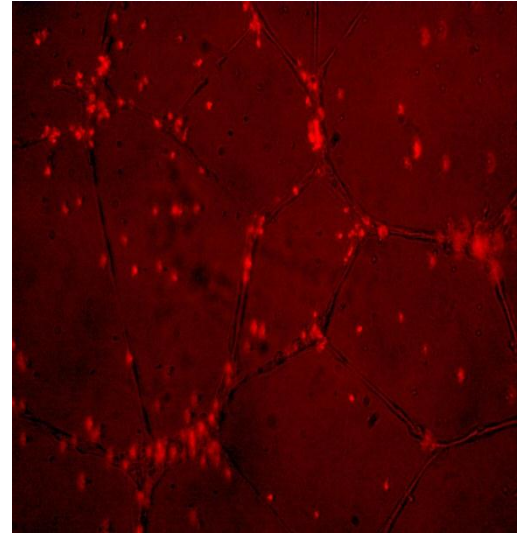


labelled with Di -I-LDL as described above were then re-plated with HUVEC (ratio of 3000 EPCs to 10000 HUVEC in 200  $\mu$ l) on top of a solidified matrigel layer and incubated at 37 °C for 20 to 24 h. Incorporation of EPC into capillary networks of HUVEC onto matrigel was examined with fluorescence microscopy. Five independent fields were assessed for each well and the mean numbers of EPCs incorporated into the HUVEC vascular networks/ x200 field were determined (figure 7-5).



***Figure 7-4: Human umbilical vein endothelial cell (HUVEC) capillary network formation on matrigel.***

*HUVEC grown overnight were treated with trypsin and re-suspended in EGM-2 at varying concentrations of 5000, 8000 and 10000 cells in 100 $\mu$ l respectively prior to being seeded onto the matrigel coated 96-well plate. The formation of HUVEC vascular networks was examined with light microscopy to establish the optimal concentration of HUVEC for capillary network formation.*

**A****B**

***Figure 7-5: Endothelial progenitor cell (EPC) incorporation into matrigel human umbilical vein endothelial cells (HUVEC) networks.***

*EPCs were labeled with Di-I-LDL and then replated with HUVEC on top of a solidified matrigel layer and incubated at 37 °C for 24 h. Incorporation of EPCs into HUVEC capillary networks was assessed by fluorescent microscopy. Five independent fields are assessed for each well, and the mean number of EPC incorporated into HUVEC vascular networks  $\times 200$  are determined. Figures 7-5A and 7-5 B display the reduced incorporation of EPCs and tubule formation (structure exhibiting length four times its width) in a patient with arterial ischemic stroke (A) compared to a healthy control child B).*

## **7.5 Statistical analysis**

Values are presented as median (range) unless otherwise specified. The Kruskal-Wallis test was used to examine overall differences in experimental laboratory markers between the study groups. Following this, statistical differences between groups were determined by Mann-Whitney U test. P-values of less than 0.05 (two sided) were regarded as significant. Statistical analysis was performed using SPSS version 16 and 17.

## **7.6 Results**

### ***7.6.1 Subjects***

EPC were identified using flow cytometry in 35 children with AIS (10 with recurrence) and compared to 20 paediatric controls. The demographics, clinical and radiological data for the subpopulation of these 35 children with AIS in whom endothelial repair responses were assessed are summarised in table 7-2. In select subjects (5 in each group) the EPC ability to form colonies and incorporate into endothelial cell vascular structures were additionally evaluated.

<b>Demographics</b>	<b>AIS recurrence n=10 (%)</b>	<b>AIS no recurrence n=25 (%)</b>	<b>P value</b>
Sex (M:F)	6:4	16:9	1
Age (median, range) years	8.2 (0.9-15.4)	9.4 (2.7-17.4)	0.681
<b>Clinical features</b>			
Focal neurological deficit	10(100%)	25 (100%)	1.0000
Diffuse neurological deficits	7 (70%)	2 (8%)	<b>0.0005</b>
<b>Sebire et al. classification of cerebral arteriopathy (Sebire et al. 2004)</b>	N=3 Arterial dissection (30%) N=1 PACNS (10%) N=1 PVA (10%) N=1 Moyamoya (10%) N= 4 Unclassified (40%)	N=16 TCA/FCA (64%) N=4 Arterial dissection (16%) N=3 PVA (12%) N=2 Moyamoya (8%)	N/a
<b>ESR mm/h (median, range) (normal range 0-10 mm/h)</b>	5.4 (1-12) mm/h	6.6 (1-28) mm/h	0.561
<b>CRP mg/L (median, range) (normal range &lt;10 mg/L)</b>	5 (3-16) mg/L	5 (3-6) mg /L	0.873
<b>MRI</b>			
Multifocal and bilateral lesions	9 (90%)	5(20%)	<b>0.0002</b>
<b>MRA</b>			
Multifocal and bilateral lesions	9 (90%)	5(20%)	<b>0.0002</b>
Anterior and posterior circulation	7 (70%)	2(8%)	<b>0.0005</b>
Collaterals	4 (40%)	2(8%)	<b>0.04</b>

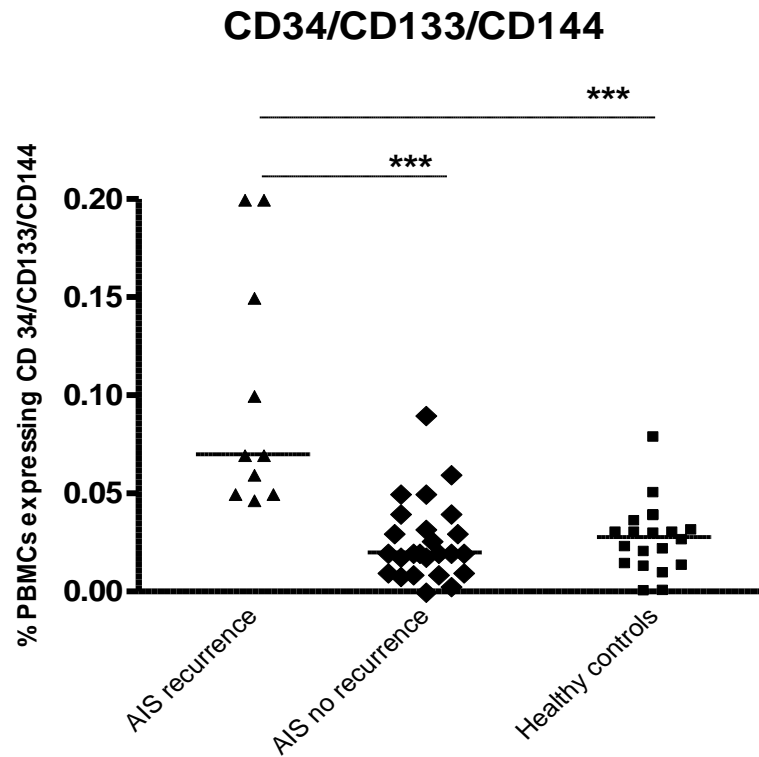
**Table 7-2: Study population characteristics.**

*Focal neurological deficits included hemiparesis, facial weakness and hemisensory loss. Diffuse neurological deficits included neurocognitive dysfunction, personality changes and concentration difficulties. M=male; F=Female; TCA=Transient cerebral arteriopathy; FCA=Focal cerebral arteriopathy; PACNS=primary angiitis of the central nervous system; ESR=erythrocyte sedimentation rate; CRP=C-reactive protein; MTHFR= Methylene-tetrahydrofolate reductase; PVA=Post varicella arteriopathy; N/a=not applicable; MRI=magnetic resonance imaging; MRA=magnetic resonance angiography. P values were calculated using Fisher's exact test for categorical data and Mann Whitney U for continuous variables. P-values of less than 0.05 (two sided) were regarded as significant.*

**7.6.2 Flow cytometric identification of EPC in children with AIS and cerebral arteriopathy**

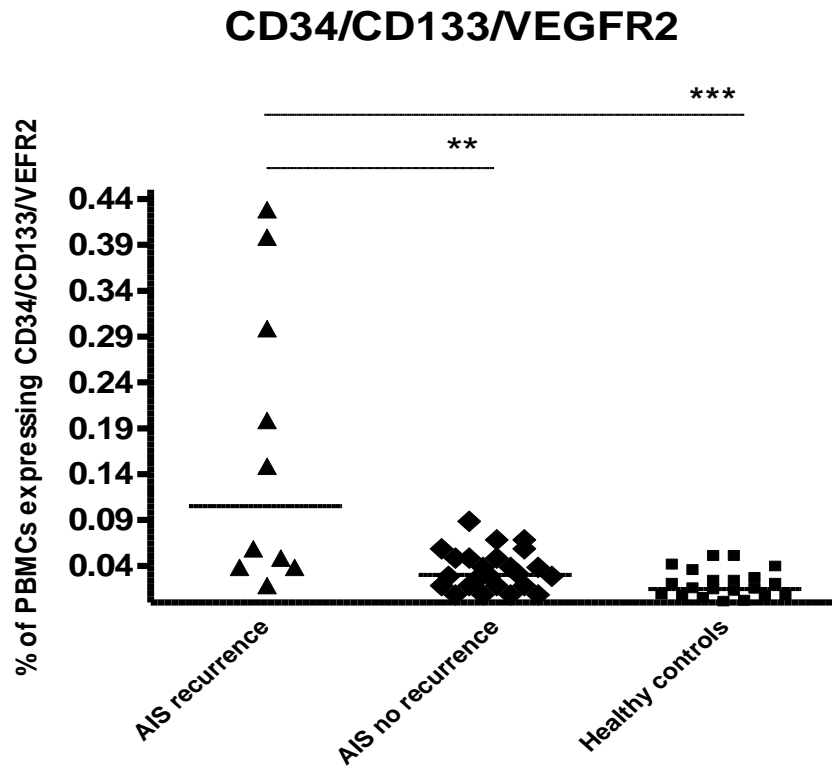
CD34/CD133/CD144 EPC were significantly raised in 10 patients with AIS recurrence median of 0.07% of PBMC gated (range 0.04-0.2 %) compared to 25 patients with no recurrence median 0.02% (0-0.09%) of PBMC gated  $p= 0.0001$  and 20 child controls median 0.03% (range 0-0.07%) PBMC gated,  $p = 0.0001$  (figure 7-5).

CD34/CD133/VEGFR2 EPC were significantly raised in 10 patients with AIS recurrence median of 0.1% of PBMC gated (range 0.02-0.4 %) compared to 25 patients with no recurrence median 0.03% (0.01-0.09%) of PBMC gated  $p= 0.005$  and 20 child controls median 0.01% (range 0.-0.05%) PBMC gated,  $p = 0.0001$  (figure 7-6).



***Figure 7-5: Circulating CD34+CD133+CD144+ endothelial progenitor cells in children with arterial ischaemic stroke (AIS) and cerebral arteriopathy.***

*CD34+CD133+CD144+ endothelial progenitor cells were significantly higher in patients with AIS recurrence compared to those with no recurrence,  $p=0.0001$  and child controls,  $p=0.0001$ . \*\*\* $P<0.0005$  with Mann Whitney U test.*

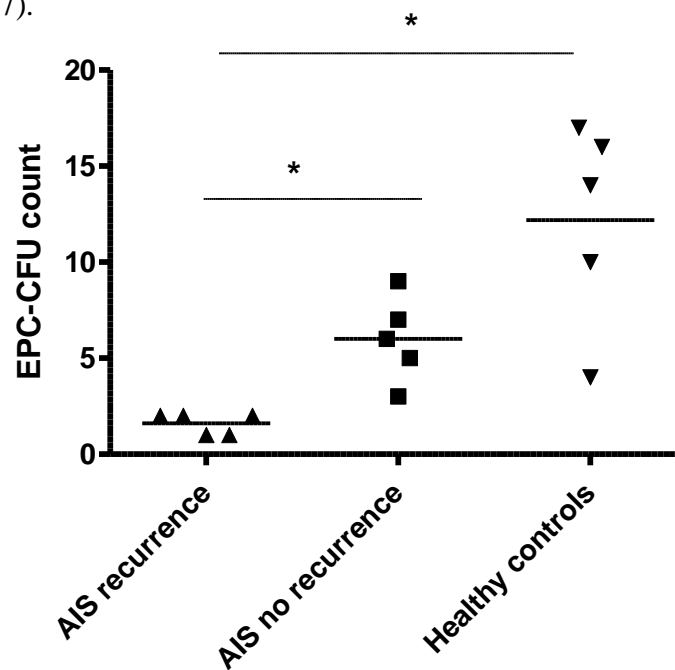


**Figure 7-6: Circulating CD34+CD133+VEGFR2+ endothelial progenitor cells in children with arterial ischaemic stroke (AIS) and cerebral arteriopathy.**

*CD34+CD133+VEGFR2+ endothelial progenitor cells were significantly higher in patients with AIS recurrence compared to those with no recurrence,  $p=0.005$  and child controls,  $p=0.0001$ . \*\*\* $P<0.0005$  with Mann Whitney U test.*

### **7.6.3 Endothelial progenitor cell- colony forming units (EPC-CFU) in children with AIS and cerebral arteriopathy**

EPC-colony forming units (EPC-CFU) were reduced in 5 children with AIS recurrence with a median 2/well (range 1-2/well) compared to 5 children with no recurrence median 6/well (range 3-9/well)  $p=0.04$  and 5 healthy control children median 14/well (range 4-17/well),  $p=0.03$  (figure 7-7).

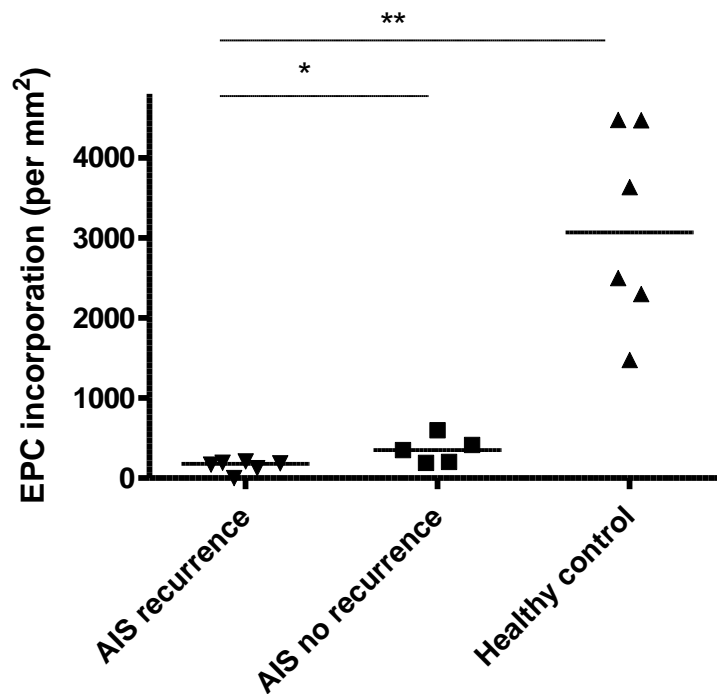


**Figure 7-7: Endothelial progenitor cell-colony forming units (EPC-CFU) in children with arterial ischaemic stroke (AIS) and cerebral arteriopathy.**



#### **7.6.4 EPC incorporation into HUVEC vascular networks for children with AIS and cerebral arteriopathy**

Incorporation of EPCs into HUVEC vascular networks in matrigel was decreased for 5 children with AIS recurrence median 124/ mm<sup>2</sup> (range 0-193/mm<sup>2</sup>) compared to 5 children with no recurrence median 307/ mm<sup>2</sup> (189-596/ mm<sup>2</sup>), p =0.022 and 5 healthy control children median 1478/ mm<sup>2</sup> (range 1478-4478/ mm<sup>2</sup>), p=0.003 (figure 7-8).



**Figure 7-8: Endothelial progenitor cell (EPC) incorporation into human umbilical vein endothelial cell (HUVEC) vascular networks.**

Endothelial progenitor cell (EPC) incorporation into human umbilical vein endothelial cell (HUVEC) capillary networks on matrigel as compared for children with AIS recurrence and those with a single event and child healthy controls. \*P<0.05 with Mann Whitney U test.

## 7.7 Discussion

Children with AIS recurrence had significantly higher levels of circulating CD34+EPC as identified with flow cytometry compared to children with a single event, but impaired EPC functional capacity: the ability of these EPC to grow in culture, form colonies and incorporate into endothelial cell vascular networks was diminished. Therefore in parallel to endothelial-injury processes reflected in an increase in CECs and endothelial derived MPs, there could be an additional disturbance in endothelial repair in children with AIS recurrence.

The balance between endothelial injury and regeneration is critical for the maintenance of vessel integrity (Sabatier et al. 2009). If baseline levels of CEC, EMP and EPC represent the physiological equilibrium of endothelium in healthy situations, any shift in this balance could be important clinically. Combining these indices of vascular injury and repair in a multi-biomarker strategy (CEC, MP and EPC) could provide a prognostic score based on endothelial phenotype for individual patients, potentially offering a novel means of assessing vascular risk and monitoring therapeutic efficacy. To date, however, little is known about the balance of endothelial injury and repair in childhood AIS. The present study is the first in this field, and demonstrates that in children with AIS recurrence there is altered endothelial repair responses, which could contribute to disease progression when considered in the context of the burden of excess endothelial injury described in preceding chapters.

The finding of elevated levels of circulating CD34+EPC as identified with flow cytometry is intriguing, and consistent with observations in other paediatric vasculitides. Mobilization of

EPCs from bone marrow in rapid response to tissue ischaemia could be an attempt to mediate vascular repair, and hence higher levels of EPCs could be reflective of the extent of the tissue injury. Consistent with this hypothesis is the observation that children with Kawasaki disease (KD) complicated by coronary artery aneurysms have higher circulating EPCs in the subacute phase of the illness than KD patients without coronary artery abnormalities, suggesting that EPC release is proportionate to the severity of the vascular injury in that scenario (Xu et al. 2010). Additionally, Clarke et al. observed that children with active systemic vasculitis mount a similar increased CD34+EPC response compared to healthy controls or children with inactive vasculitis, although the functional properties of EPC were not assessed in that study (Clarke et al. 2010). Interestingly, in conditions of acute vascular ischaemia and endothelial injury, an increase in the number of circulating CD34+/KDR+ cells has been observed in association with a concomitant increase in levels of mobilizing factors, such as VEGF and SDF-1, suggesting a causal relationship (Rabelink, de Boer, & van Zonneveld 2010). In addition recent studies proposed that CD34+/KDR+ cells are generated from CD34+cells at platelet rich sites of vascular injury thus reflecting the plasticity of CD34+cells and their capacity to respond to environmental cues (De Boer et al. 2006; De Boer et al. 2011). In chapters 3 and 4 it was established that markers of endothelial injury and platelet activation are elevated in childhood AIS recurrence. These data would now suggest that the CD34+/KDR+ cell fraction increases in response to this vascular injury and platelet activation, perhaps representing an attempt to recruit to the injured vessel progenitor cells that are able to modulate vascular repair.

In contrast to the elevated CD34+EPCs, the colony-forming capacity and ability of EPC to incorporate into endothelial networks was impaired in children with recurrent AIS. This observation underscores the functional differences in cellular populations with potential to influence angiogenic responses, and is in keeping with observations in adults with rheumatoid arthritis (RA)(Egan et al. 2008). Egan et al. observed that whilst circulating levels of CD34+EPCs were unaffected in RA, colony-forming capacity was significantly reduced (Egan et al. 2008). Thus it is possible that there is functional impairment of EPC responses in children with recurrent AIS, despite attempts to generate a reparative response (increased circulating CD34+ EPCs) in children with recurrent AIS, a situation observed in other systemic inflammatory diseases. In RA it is suggested that impaired colony-forming capacity is a consequence of chronic systemic inflammation and an unfavourable inflammatory milieu (Egan et al. 2008). Children with AIS, however, are conspicuous by the lack of apparent systemic inflammation, raising the possibility that local vascular inflammation in the central nervous system may be enough to adversely influence functional EPC responses. Alternatively, children with recurrent AIS may have subclinical levels of systemic inflammation not detected by routine assessment of acute phase reactants.

Another important implication of impaired EPC function in children with recurrent AIS could relate to the influence of EPCs on neurogenesis, the process of generating neural cells from their progenitors (Eriksson et al. 1998). EPCs may promote a favourable angiogenic environment that facilitates effective neurogenesis after cerebral ischaemia (Eriksson et al. 1998). Palmer et al. proposed the idea of a vascular niche based on the hypothesis that there is co-development of neurogenesis and vasculogenesis in neural tissue (Palmer et al. 2000).

Endothelial cells are now known to release factors or chemokines, such as brain-derived neurotrophic factor (BDNF) which stimulates the regeneration of both embryonic and adult neural stem cells and also promote the neuronal function (Shen et al. 2004a). Since EPCs are the precursors of endothelial cells, they may have the potential to promote neurogenesis after cerebral ischemia by providing a favourable environment for new neural cells through their paracrine effects (Shen et al. 2004b; Taguchi et al. 2004; Zhang et al. 2002). Taguchi et al. for instance administered human CD34+EPCs, to immunocompromised mice 48 h after stroke, and observed enhanced neovascularization at the border of the ischaemic zone, and endogenous neurogenesis (Lapergue, Mohammad, & Shuaib 2007; Taguchi et al. 2004). This neurogenesis was suppressed by an antiangiogenic agent (endostatin) and increased by a proangiogenic agent (erythropoietin), demonstrating the importance of an angiogenic environment to neurogenesis after stroke (Lapergue, Mohammad, & Shuaib 2007; Taguchi et al. 2004). Further studies to establish the exact relationship between angiogenesis and neurogenesis and the potential relevance to paediatric AIS are clearly needed.

Longitudinal studies of EPC changes with time after the initial cerebrovascular event and in response to therapy would be of considerable interest in paediatric AIS and could enhance our understanding of EPC biology in this context. Overall interpretation of these preliminary novel observations remains challenging, however, as the true identity and phenotype of EPCs remains ill-defined and needs to be further explored. The initial observations made in this thesis are intriguing, however, and suggest that it may be possible to explore the endothelium non-invasively by defining an imbalance between endothelial damage and repair capacity for children with AIS. This multimarker approach combining endothelial

injury and repair indices to provide an overall 'endothelial index' could provide important prognostic information in relation to risk of recurrent AIS in children. Importantly, therapeutic strategies aiming to improve endothelial function could target the selective reduction of endothelial injury, or the promotion of regenerative mechanisms. At the individual level, such personalised therapeutic options could be tailored depending on the relative severity of injury and/or impaired repair to prevent AIS recurrence. Future studies are therefore needed to further understand the relevance of EPCs in AIS, including better isolation methods, and in-depth studies regarding the potential interplay between angiogenesis and neurogenesis.

## **8 General discussion and future directions**

### **8.1 Summary: endothelial injury and repair in childhood arterial ischaemic stroke (AIS) and cerebral arteriopathy**

Childhood AIS causes significant lifelong morbidity and mortality (Ganesan et al. 2002). Abnormalities of the cervical or intracranial circulation, termed arteriopathies are the leading mechanism of both cause and recurrence of childhood AIS but an incomplete understanding of pathophysiology has hindered the development of targeted therapies or prevention strategies (Ganesan et al. 2003; Mackay et al. 2011). Though it may represent one or multiple different diseases, a distinct pattern of large vessel arteriopathy is most commonly observed in otherwise healthy school aged children presenting with childhood AIS (Amlie-Lefond et al. 2009; Benseler et al. 2006; Braun et al. 2009; Chabrier et al. 1998; Mackay et al. 2011). Many different terms have emerged to describe this syndrome, some of which directly imply an infectious or inflammatory mechanism: transient cerebral arteriopathy (TCA); focal cerebral arteriopathy (FCA); post varicella arteriopathy (PVA); and childhood primary angiitis of the central nervous system (cPACNS) (Amlie-Lefond et al. 2009; Benseler et al. 2006; Braun et al. 2009; Chabrier et al. 1998; Mackay et al. 2011). Of note, inferring an inflammatory pathophysiology has direct and immediate therapeutic implications with readily available anti-inflammatory and/or immunosuppressive medications. Confirming the inflammatory nature of childhood cerebral arteriopathies is challenging however because clinical presentation may be diverse, and there is an ever expanding list of genetic noninflammatory vasculopathies being discovered (Munot, Crow,

& Ganesan 2011a). Even for those cases where there is a true inflammatory vasculitic component to the vasculopathy, conventional acute phase reactants such as ESR and CRP are insensitive; CSF biomarkers thus far have proven to be non-specific; and biopsy of arteries and/ or brain/meningeal tissue to confirm vasculitis is rarely performed, and lacks sensitivity due to the patchy nature of the disease in any case (Benseler et al. 2006; Cellucci, Tyrrell, Sheikh, & Benseler 2011; Elbers et al. 2010). Imaging techniques, while steadily improving, may be normal or non-specifically abnormal (Aviv et al. 2006; Eleftheriou et al. 2010). Therefore, there is an urgent need for more specific, non-invasive and sensitive diagnostic biomarkers for children with AIS, to be used in conjunction with imaging findings to provide: (i) novel insight into disease pathophysiology, especially the role of endothelial injury, inflammation and thrombosis; (ii) and to help guide clinical care including appropriate treatment options, facilitate prognostication, and to monitor over time. On the background of these challenges, this thesis explored novel biomarkers of endothelial injury/repair and hypercoagulability and compared patients with recurrent clinical disease course, to those children with a single event.

A number of methods for detecting endothelial cell components allowing assessment of the molecular events associated with vascular injury were employed in this study: cellular MPs, and whole circulating endothelial cells (CECs) in blood. CEC effectively differentiated between children with recurrent AIS and those with a single event despite a wide range of radiological and clinical presentations. This approach was complimentary to current radiological and clinical predictors of AIS recurrence, and suggests that persistent endothelial injury is a feature of those with recurrent stroke. Consistent with this novel



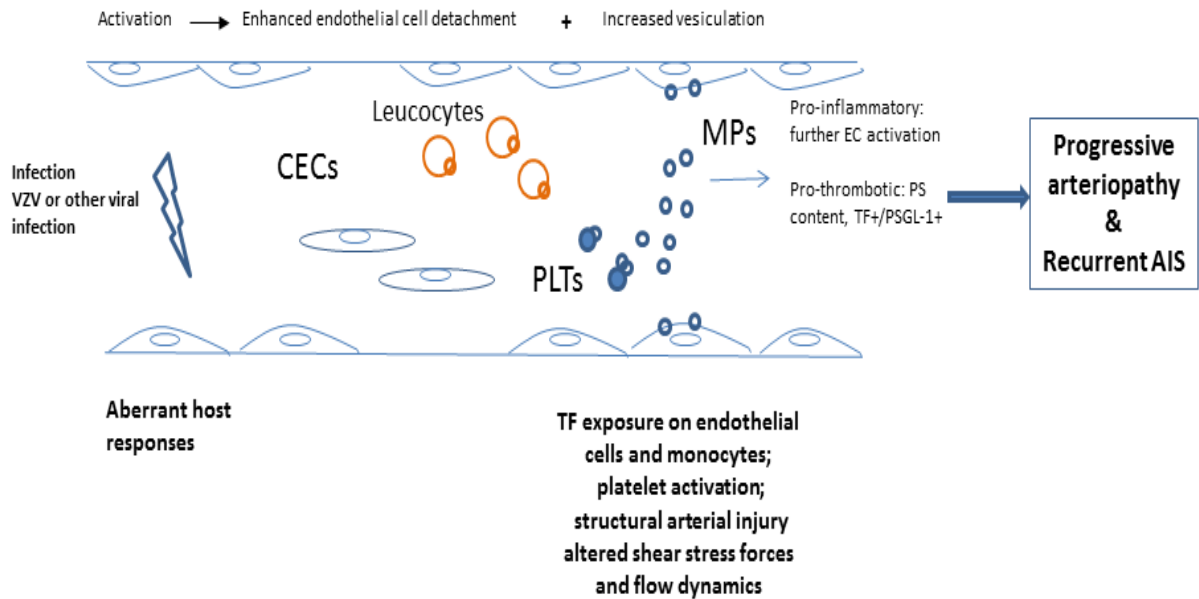
observation, children with recurrent AIS had evidence of increased levels of total (AnV+) MPs; and in particular MPs of endothelial, platelet, neutrophil and monocyte origin. Notably these MPs were highly prothrombotic, mainly due to PS exposure providing a platform for the assembly and activation of coagulation factors; but also due to expression of TF on some MP.

An efficient *in vitro* assay to assess MP-related hypercoagulability by quantifying MP-mediated thrombin generation was developed and is discussed in chapter 5. Thrombin is the key product of the coagulation system and is produced following activation of factor X by tissue factor/factor VIIa (Hemker, Wielders, Kessels, & Beguin 1993). Measurement of thrombin generation, and in particular the enzymatic work potential of thrombin, provides a method for quantifying the composite effect of the multiple factors that determine coagulation capacity and the influence of the environment on these factors (Bidot et al. 2008; Hemker, Wielders, Kessels, & Beguin 1993; Hemker et al. 2003). Enhanced MP-mediated thrombin generation was higher in systemic vasculitis patients with thromboembolic events. A similar approach was then used to determine if the same was true in children with AIS by assessing the pro-coagulant properties of MPs in children with recurrent AIS compared to those with a monophasic disease course. Significant differences in peak thrombin, endogenous thrombin potential, velocity index and lag time were demonstrated between these patient groups and controls, confirming an increased prothrombotic tendency associated with re-infarction in these patients. Overall the observations made in this thesis suggest that a state of chronic endothelial activation and injury (increased CECs and EMPs), platelet activation (increased platelet MPs), and increased MP mediated thrombin generation could be important determinants of AIS recurrence, providing important links between

inflammation and infection, leucocyte, platelet and endothelial activation, and pathological procoagulant activity in children with AIS (summarised in figure 8-1).

In addition, a disturbance in EPCs potentially important for endothelial repair in children with AIS recurrence was demonstrated in chapter 7. The ability of these progenitor cells to grow in culture, form colonies and incorporate into endothelial cell vascular networks was impaired. The clinical relevance of this observation remains uncertain, however, but could be compatible with further compromise of endothelial integrity and hence adverse prognostic outcome, as has been observed in adults with atherosclerosis (Hill et al. 2003; Werner et al. 2005).

There are a number of limitations to the data presented in this thesis many of which have already been discussed in the relevant results chapters. This chapter will highlight these limitations, and areas of further research that could address some of the unanswered questions will be discussed. Limited preliminary data will be presented to illustrate the sort of approaches that may be used in these future studies.



**Figure 8-1: Circulating endothelial cells and cellular microparticles in childhood arterial ischaemic stroke (AIS) recurrence.**

*One or more widely distributed infectious agents evoke an abnormal immunological response in genetically susceptible individuals, leading to vascular inflammation, cerebral endothelial injury. As a result whole endothelial cells are detached (circulating endothelial cells-CECs) and can be detected in the peripheral blood. In addition there is increased endothelial, leucocyte and platelet membrane vesiculation resulting in release of endothelial, platelet microparticles, and leucocyte (MPs). These MPs may induce further endothelial cell activation, and are prothrombotic due to phosphatidylserine (PS) and tissue factor content. In addition, TF+ MPs also express PSGL-1 responsible for binding of these TF+MP to activated platelets expressing P-selectin at the thrombus edge, and hence can contribute to clot propagation. Thus inflammation and infection causes cellular and platelet activation, and endothelial activation/injury. Increased MP release resulting from this inflammatory activation, leads to pathological MP-mediated thrombin generation, with possible for the contribution to AIS recurrence in those where these processes persist.*

## **8.2 Biomarker specificity for childhood AIS and cerebral arteriopathy**

Apart from non-atherosclerotic arteriopathies, multiple other risk factors such as cardiac disease are commonly identified in childhood AIS (Ganesan et al. 2003; Mackay et al. 2011). Several other neuroinflammatory disorders may also mimic stroke in childhood. Demyelinating disorders such as acute demyelinating encephalomyelitis (ADEM) and multiple sclerosis (MS) may be difficult to differentiate from small vessel arteriopathies causing AIS on clinical and neuro-imaging features alone (Cellucci & Benseler 2010; Elbers & Benseler 2008). There are also a number of newly recognised neuronal antibody-associated inflammatory brain diseases triggered by the binding of antibodies to cell surface receptors, channels, or enzymes in the CNS that have with similar neurological presentations, and indistinguishable radiological findings mimicking AIS (Vincent et al. 2011). These include: N-methyl-D-aspartate (NMDA) receptor antibody associated encephalitis, neuromyelitis optica (NMO), voltage-gated potassium channels (VGKCs), glutamic acid decarboxylase (GAD), GluR1/2 alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and gamma-aminobutyric acid (GABAB) receptors antibody associated encephalitides (Vincent et al. 2011). The role of inflammation and endothelial injury in these conditions is currently poorly defined. Comparing circulating endothelial MP profiles and CEC in children with cardiac disease related stroke, MS, other neuroinflammatory disorders and childhood arteriopathic AIS would be of considerable interest and would help establish the specificity of our findings.

### **8.3 Prognostic value of studied biomarkers-need for prospective studies**

Whilst the preliminary prospective data presented herein would support the proposal that these markers may have important predictive value for identification of those children with high risk of stroke recurrence, the preliminary nature and the possibility that these observations represent epiphenomena is emphasized. Multicentre prospective validity studies to confirm these observations are now needed, a significant challenge since paediatric AIS is rare, and the assays required and described in this thesis are non-routine. Longitudinal studies will also allow us to study the effect of treatment on the levels of these biomarkers.

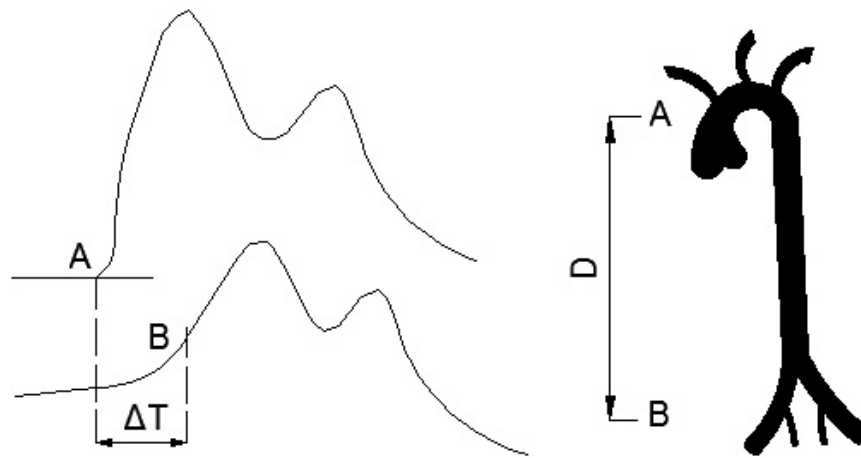
### **8.4 Pulse wave velocity-assessing arterial stiffness**

Arterial stiffness is one of the earliest detectable manifestations of adverse structural and functional changes within the vessel wall (Arnett et al. 1994 ;Blacher et al. 2001; Laurent et al. 2006; Weber et al. 2004). It is now widely recognised that changes in the arterial wall as early as in childhood are associated with altered levels of cardiovascular risk factors and eventually impact on longer term cardiovascular morbidity and mortality (Berenson et al. 1998; Tounian et al. 2001). As a result, there is increasing interest in the determinants of the initiation and progression of early arterial disease and the potential for reversibility and prevention from a young age (Berenson et al. 1998; Tounian et al. 2001). Pulse wave

velocity (PWV) is the most validated method to noninvasively quantify arterial stiffness (Asmar et al. 1995; Barac et al. 2007; Laurent et al. 2006; Wilkinson et al. 2002). It is considered the gold standard index of arterial stiffness, given that it is simple, accurate, reproducible, and strongly predicts adverse outcomes (Lehmann 1999; Millasseau et al. 2005; Sutton-Tyrrell et al. 2005). PWV can be determined by measuring the pulse transit time from the pressure waveforms at the 2 sites along a vascular segment (figure 8-2) (Laurent et al. 2006; Lehmann 1999; Millasseau et al. 2005; Sutton-Tyrrell et al. 2005; Wilkinson et al. 2002). The distance (D) is divided by the wave foot-to-foot time it takes for that forward wave to reach the end measuring point. PWV is inversely related to vascular compliance (Laurent et al. 2006; Lehmann 1999; Millasseau et al. 2005; Sutton-Tyrrell et al. 2005; Wilkinson et al. 2002). Hence, a stiffer vessel will conduct the pulse wave faster than a more distensible and compliant vessel. A number of previous studies have demonstrated that PWV obtained by non-invasive automatic devices is not only a marker of vascular damage but also a prognostic predictor of cardiovascular events and cardiovascular mortality in patients with different co-morbidities (Cheung et al. 2007; Lehmann 1999; Munakata et al. 2003; Sutton-Tyrrell et al. 2005). In children Cheung et al. have previously shown an increased PWV and hence decreased distensibility in the peripheral arterial segment in children with polyarteritis nodosa amplified during acute inflammatory exacerbation (Cheung et al. 2004; Cheung, Wong, & Ho 2007). This finding suggests that chronic vasculitis leads to a significantly reduced distensibility, the magnitude of which is amplified during the acute inflammatory stage of the disease (Cheung, Ho, Tam, & Yung 2004; Cheung, Wong, & Ho 2007).

The predictive value of arterial stiffness specifically for adult stroke was demonstrated in a longitudinal study that included 1715 patients with essential hypertension and measurements of carotid-femoral PWV at entry (Laurent et al. 2003). Over a mean follow-up period of 7.9 years, during which 25 fatal strokes occurred, PWV significantly predicted stroke (relative risk = 1.39 [(95% CI 1.08, 1.72];  $p = 0.02$  for each 4 m/sec increase) independently of classical cardiovascular risk factors, including age, cholesterol level, diabetes mellitus, smoking and mean blood pressure (Laurent et al. 2003).

Childhood AIS is commonly associated with non atheromatous arteriopathies confined to the CNS (Ganesan et al. 2003; Mackay et al. 2011). As such one would not expect any effect on central arterial segmental elasticity or changes in systemic arterial stiffness in children affected by this condition. To date however no study has explored whether children with AIS have a secondary sub-clinical systemic effect on the peripheral vasculature resulting in impaired arterial distensibility and maybe contributing to their long term cardiovascular morbidity. Future studies are now needed to explore this hypothesis.



**Figure 8-2: Pulse wave velocity measurement.**

*A=wave recorded by the proximal transducer; B=wave recorded by the distal transducer;  
 $\Delta T$ =time delay between the foot waves; D=distance traveled by the wave.*

## **8.5 Phenotype of circulating endothelial cells (CEC)**

The phenotype of CEC was not examined in this thesis. Several lines of evidence suggest that CECs could also be pro-inflammatory (Kirsch et al. 2007; Solovey et al.1997). Under physiological conditions dying or dead cells are rapidly removed by macrophages or by neighbouring cells before their inflammatory potential can unfold (Henson et al. 2001). CECs may be present in high numbers in certain vascular disorders and therefore overwhelm the clearance mechanisms thus initiating a series of signalling processes and thereby gaining pathogenic importance. Kirsch et al. were able to demonstrate that apoptotic and necrotic endothelial cells and their fragments are rapidly internalized by healthy endothelium (Kirsch



et al. 2007). The authors also showed that endothelial cells exposed to apoptotic and necrotic cells exhibit enhanced adhesion properties for leukocytes, and that isolated CECs from patients with vasculitis aggravated this (Kirsch et al. 2007). These effects on binding properties could be explained, at least in part, by the release of the pro-inflammatory chemo-attractants IL-8 and MCP-1 (Kirsch et al. 2007). Cytokine profiles may differ in various vascular disorders, and could therefore influence interactions between CECs and healthy endothelium. These interactions may also be influenced by the nature of neighbouring endothelial cells (microvascular versus macrovascular). As endothelial cells participate in the homeostasis of coagulation, detachment of these cells and loss of endothelial integrity is likely to significantly impact on haemostasis (George et al. 1993; Sabatier et al. 2009; Solovey et al. 1997). George et al. reported that some CECs contain thrombomodulin, but whether or not this molecule is still bio-active is unclear (George et al. 1993). Solovey et al. found that CECs in sickle cell disease expressed tissue factor that was functionally active (Solovey et al. 1997). Therefore CEC, in addition to providing a useful biomarker of endothelial injury, could also be important pathogenic mediators. Even though several lines of evidence suggest that CEC can amplify endothelial injury and inflammation, the cross sectional nature of my study precludes any causal effect to be determined. Future studies to address the hypothesis that these reported biomarkers are indicative of persistent vascular inflammation leading to further vascular damage, and not just an epiphenomenon/consequence of stroke (and associated endothelial injury) are now needed.

## **8.6 Microparticles in cerebrospinal fluid (CSF)**

It could also be important to investigate whether MPs are present in the cerebrospinal fluid (CSF) of children with AIS, possibly also resulting in enhanced thrombogenicity. Direct sampling of CSF could provide more sensitive detection of pathophysiological processes in paediatric AIS and cerebral arteriopathy. Morel et al. have previously shown that flow cytometric identification and enumeration of MPs in CSF is possible in patients with traumatic brain injury (Morel et al. 2008a). The presence of cellular MPs in the CSF may reflect injury of the blood-brain barrier, which could have prognostic significance for identifying patients at risk of AIS recurrence. These CSF MPs (if present) could also prove useful for the study of neuronal or vascular cell activation/apoptosis.

## **8.7 Microparticles as shuttles of mRNA and microRNA**

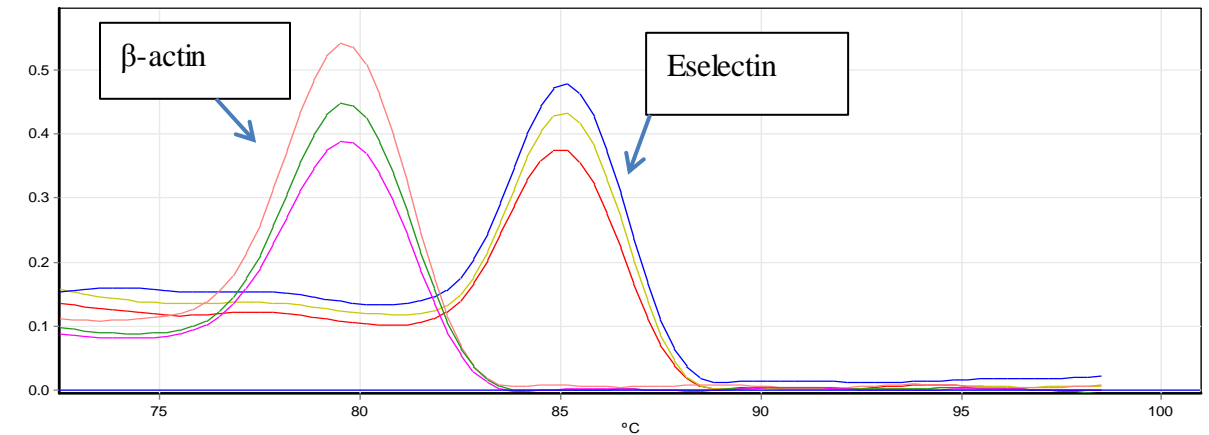
MPs represent a novel mechanism of intercellular communication mediating inflammation and coagulation (Morel et al. 2006; Sabatier et al. 2009). Their effect on target cells was long attributed to their specific lipid composition, as well as to intravesicular chemokines (Morel, Toti, Hugel, & Freyssinet 2004; Morel et al. 2006; Sabatier et al. 2009). The hypothesis that MP can also affect protein expression of their target cells by delivering RNAs is intriguing. Preliminary observations suggest that MP contain mRNA. As depicted in figure 8-3. EMP harvested from supernatants of HUVEC stimulated with TNF- $\alpha$  100 ng/ml for 4 hours expressed E-selectin mRNA.

These preliminary findings suggest that MPs could facilitate genetic exchanges between different tissues within the body. This may have implications for cell activation, phenotypic modification, and reprogramming of cell function. MP of different origin may contain different subsets of mRNA and different cell types may be specifically targeted by plasma MP. In addition, exploring the mRNA content of MPs may help us establish the anatomic origin of circulating MPs. In children with childhood AIS we confirmed the presence of endothelial derived EMP based on surface protein expression, but whether these are released from the cerebral endothelium remains uncertain. Kallman et al. have previously shown that there are significant differences in the mRNA profile of human cerebral microvascular endothelial cells and HUVEC (Kallmann et al. 2002). Taking this into account it is tempting to speculate that MP released from different vascular beds will contain mRNA indicative of their endothelial cell of origin.

In addition, recent progress in the understanding of microRNA prompts the question of whether MP also affect their target cells via transferring endogenous microRNAs and whether these MP have different microRNA patterns than their maternal cells. MicroRNAs are a class of approximately 22 nucleotide noncoding RNAs that mediate post-transcriptional gene regulation by binding to and repressing specific messenger RNA targets (Bartel 2004). MicroRNAs are present in the human circulation in a cell-free form and are remarkably stable in plasma despite high circulating RNase activity (Bartel 2004). To date, 587 human microRNAs have been identified. A single microRNA can modulate several genes, and a single gene can be modulated by several microRNAs: about 10–30% of human genome expression is modulated by microRNAs (Bartel 2004). MicroRNAs play a critical role in the

control of: metabolism, cell-differentiation, cell-proliferation and cell-apoptosis (Bartel 2004). Circulating microRNAs have also recently emerged as biomarkers for cardiovascular diseases (Lu et al. 2008; Small et al. 2010). Experiments to confirm the presence of microRNA in MP are ongoing at the time of writing this thesis.

MPs as shuttles of microRNA may also play a significant role in either the promotion or inhibition of angiogenesis. Parallel microRNA and mRNA expression profiling in angiogenic-associated diseases in combination with functional studies on ECs and large-scale proteomics could help us understand the complex microRNA-mediated gene regulatory networks in angiogenesis.



**Figure 8-3: Messenger RNA content of microparticles.**

RNA for PCR was extracted using Trizol (Invitrogen). The total amount of RNA in the microparticle preparations was determined spectrophotometrically. cDNA was reverse-transcribed from 1  $\mu$ g of total RNA by use of a commercial reverse transcription system (Applied Biosystems). Real-time quantitative PCR for  $\beta$ -actin (Quantitect, Qiagen) and E-selectin was performed on a 6000 Rotor Gene Sequence Detection System (Qiagen) using SYBR-Green (SYBR PCR Master Mix, Applied Biosystems). The following Eselectin primers were synthesised by Invitrogen: CCC AGA GCC TTC AGT GTA CC (forward) and CAG AGC CAT TCT GAG GCT GG (reverse). PCR conditions were: 95°C for 15 min, followed by 40 cycles of 94°C for 15 sec, 55°C for 20 secs and 72°C for 20s. Each reaction was performed in triplicate to ensure reproducibility. A standard curve (using standard cDNA extracted from HUVEC) was produced for each gene to calibrate the quantitation. Target PCR efficiency was between 95-100% and correlation coefficient for the standard curve was greater than 0.9. Melt curves for  $\beta$ -actin and E-selectin on endothelial derived MPs are shown. The x axis displays temperature and the y axis change in fluorescence.

## **8.8 VZV and cerebral arteriopathy in childhood arterial ischaemic stroke (AIS)**

Strong epidemiological data link VZV infection to FCA and childhood AIS (Askalan et al. 2001; Lanthier, Armstrong, & Domi 2005; Sebire, Meyer, & Chabrier 1999). Nagel et al. have recently suggested that stroke in VZV vasculopathy may result from changes in arterial caliber and contractility produced in part by abnormal accumulation of smooth muscle cells and myofibroblasts in thickened neointima and disruption of the media (Nagel et al. 2011). Analysis of the morphology and composition of the thickened intima and media, and the location of viral antigen in the adventitia in early VZV vasculopathy, revealed clues to the possible mechanisms of VZV induced vascular remodelling that leads to arteriopathy and AIS (Nagel et al. 2011). After vascular injury adventitial fibroblasts can differentiate into myofibroblasts that proliferate and migrate to the intima (Nagel et al. 2011). These cells may secrete factors that create a proinflammatory environment, further contributing to vascular wall remodelling. Also they may interact with medial smooth muscle cells influencing their phenotype. These observations suggest that perhaps human cerebral adventitial fibroblasts may be important in the pathophysiology of childhood AIS (Nagel et al. 2011; Sartore et al. 2001). Alternatively, adventitial dendritic cells have been shown to become activated and contribute to a proinflammatory environment leading to vascular wall remodelling in other cerebrovascular diseases (Inder et al. 2000). Studies of dendritic cell activation in VZV-infected arteries would thus be of considerable interest.

## **8.9 Paracrine effects of bone marrow derived progenitor cells contributing to angiogenesis and neurogenesis**

Bone marrow derived progenitor cells are believed to augment neo-vascularization not only as incorporating cells into newly developing capillaries, but also in a paracrine fashion through the secretion of angiogenic growth factors (Asahara et al. 1999b; Hirschi, Ingram, & Yoder 2008; Rehman, Li, Orschell, & March 2003; Richardson & Yoder 2011). Recent evidence also suggests that brain repair involves a collection of natural mechanisms that are activated following stroke, including angiogenesis and neurogenesis (Palmer, Willhoite, & Gage 2000; Taguchi et al. 2004). It has been demonstrated that endothelial repair can promote neurogenesis by creating a favorable environment for neuroplasticity, leading to a beneficial impact on infarct volume, cell death and perhaps most importantly on functional recovery (Palmer, Willhoite, & Gage 2000; Taguchi et al. 2004). Whether the ability of circulating progenitor cells to secrete angiogenic and neurogenic support factors is impaired or not is unknown. If indeed this pathway is dysregulated, further understanding of this mechanism could translate into the design of new agents for prevention and treatment of stroke in children.

## Appendix 1

**The CASCADE Criteria Acute Primary Classification: Anatomic Features (adapted from Bernard et al., 2012).**

CASCADE= Childhood AIS Standardized Classification and Diagnostic Evaluation;  
 AIS=Arterial ischaemic stroke; CTA=computed tomography angiography; MRA=magnetic resonance angiography.

<b>Primary Classification– Select One Only Definition</b>	
Small-vessel arteriopathy of childhood	<p>a. Definitive–Confirmation of the definitive diagnosis of small-vessel arteriopathy of childhood requires multifocal arterial narrowing of small-caliber vessels on conventional angiogram and evidence of small-vessel arteriopathy on biopsy, including evidence of intramural/vasocentric inflammation of the small muscular arteries, capillaries, and/or venules on brain biopsy. Supportive evidence can be obtained from electron microscopy demonstrating endothelial cell activations and/or tubular reticular inclusions. Perivascular demyelination and/or gliosis can be found; however, specific histological features of other inflammatory brain diseases of childhood must be absent (ie, diffuse parenchymal demyelination).</p> <p>b. Radiographic–Confirmation of the radiographic-proven diagnosis of small-vessel arteriopathy of childhood requires multifocal arterial narrowing of small-caliber vessels on conventional angiogram.</p> <p>c. Biopsy–Confirmation of the biopsy-proven diagnosis of small-vessel arteriopathy of childhood requires evidence of small-vessel arteriopathy on biopsy, including evidence of intramural/vasocentric inflammation of the small muscular arteries, capillaries, and/or venules on brain biopsy. Supportive evidence can be obtained from electron microscopy demonstrating endothelial cell activations and/or tubular reticular inclusions. Perivascular demyelination</p>



	<p>and/or gliosis can be found; however, specific histological features of other inflammatory brain diseases of childhood have to be absent (ie, diffuse parenchymal demyelination).</p> <p>d. Probable–Suspected small vessel arteriopathy is based upon a small vessel distribution of infarct (without another identified aetiology), non-invasive imaging, and/or a known disease process associated with small-vessel arteriopathy (ie, meningitis or lupus).</p>
<p>2. Unilateral focal-cerebral arteriopathy of childhood (FCA)</p>	<p>a. Anterior circulation with collaterals (would include some types of possible moyamoya disease and some patients with progressive primary angiitis of the central nervous system of childhood ).–Confirmation of the diagnosis requires MRA, CTA or computed angiography (CA) displaying both (1) unilateral stenosis or vessel irregularity of a large intracranial artery (internal carotid artery, middle cerebral artery, anterior cerebral artery) supplying the territory of infarct, and (2) evidence of an excessive collateral network of vessels distal to the occluded artery.</p> <p>b. Anterior circulation without collaterals (would include conditions such as transient-cerebral arteriopathy, post-varicella arteriopathy, and large-vessel childhood primary angiitis of the central nervous system). Confirmation of the diagnosis requires MRA, CTA, or CA displaying both (1) unilateral stenosis or vessel irregularity of a large intracranial artery (internal carotid artery, middle cerebral artery, anterior cerebral artery) supplying the territory of infarct, and (2) no evidence of excessive collateral network of vessels distal to the occluded artery.</p> <p>c. Posterior circulation (would include conditions such as basilar artery stenosis)–Confirmation of the diagnosis of focal-cerebral arteriopathy within the cerebral posterior circulation requires MRA, CTA or CA displaying unilateral stenosis or vessel irregularity of a large intracranial artery (posterior cerebral artery, basilar or vertebral) supplying the territory of infarct and not meeting definition of dissection.</p> <p>d. Other–such as congenital anomaly.</p>
<p>3. Bilateral cerebral</p>	<p>a. With collaterals (would include conditions such as</p>

<p>arteriopathy of childhood</p>	<p>moyamoya disease or Fibromuscular dysplasia)–Confirmation of the diagnosis requires MRA, CTA, or CA showing (1) bilateral stenosis or vessel irregularity of a large intracranial artery (internal carotid artery, middle cerebral artery, anterior cerebral artery, posterior cerebral artery) supplying the territory of infarct, and (2) evidence of excessive collateral network of vessels distal to the occluded arteries.</p> <p>b. Without collaterals (would include some types of possible moyamoya disease)–Confirmation of the diagnosis requires MRA, CTA, or CA showing (1) bilateral stenosis or vessel irregularity of a large intracranial artery (internal carotid artery, middle cerebral artery, anterior cerebral artery, posterior cerebral artery) supplying the territory of infarct, and (2) no evidence of excessive collateral network of vessels distal to the occluded arteries.</p> <p>c. Other–such as congenital anomaly.</p>
<p>4.Aortic/Cervical Arteriopathy</p>	<p>a. Dissection–Confirmation of the diagnosis of intracranial- or cervical-arterial dissection requires CTA, magnetic resonance imaging/MRA, or CA with 1 of the following 3 patterns: (1) angiographic findings of a double-lumen, intimal flap, or pseudo aneurysm, or, on axial T1 fat saturation magnetic resonance imaging images, a “bright crescent sign” in the arterial wall; (2) the sequence of cervical or cranial trauma, or neck pain, or head pain less than 6 weeks preceding angiographic findings of segmental arterial stenosis (or occlusion) located in the cervical arteries; (3) angiographic segmental stenosis (or occlusion) of the vertebral artery at the at the level of the C2 vertebral body, even without known traumatic history. (Adapted from Sebire and colleagues, 2004)</p> <p>b. Takayasu arteritis–Confirmation of the diagnosis of Takayasu arteritis requires angiographic abnormalities (CA, CTA, or MRA) of the aorta or its major branches (mandatory criterion) plus at least 1 of the following 4 features:</p> <ul style="list-style-type: none"> <li>– Decreased peripheral artery pulse(s) and /or claudication of the extremities</li> <li>– Blood pressure difference <math>\geq</math>10mm Hg</li> <li>– Bruits over aorta or its major branches</li> </ul>

	<p>– Hypertension (related to childhood normative data)</p> <p>c. Other—such as congenital anomaly or cervical Fibromuscular Dysplasia</p>
5. Cardio- embolic	<p>a. Definite—High-risk for cardiac source of cerebral embolism (such as congenital heart disease with abnormal cardiac function, arrhythmia, or endocarditis) or cardiac procedure within 30 days of stroke and territory of large/medium-sized cerebral artery or &gt;1 arterial territory, may be large and/or haemorrhagic.</p> <p>b. Probable—_1 arterial territory, may be large and/or haemorrhagic in a child without another identifiable aetiology and 1 of the following:</p> <ol style="list-style-type: none"> <li>1) patent foramen ovale with right-to left shunt or other subtle cardiac anomaly;</li> <li>2) Occlusion: a discrete and abrupt blockage of an artery consistent with a clot, without any surrounding irregularity or stenosis suggestive of arteriopathy. (Modified from Wraige et al, 2005; and Ay et al, 2007)</li> </ol>
6. Other	<p>a. Undetermined aetiology—aetiology unclear despite complete workup (including echocardiogram MRI, and vascular imaging of head and neck)</p> <p>b. Other (ie, other location of identifiable disease that cannot be classified)</p>
7. Multi-factorial	<p>&gt;1 anatomic site of disease (ie, patients who have &gt;1 site of the primary classifications and in whom we are unable to determine the predominant site of disease)</p>

## **Appendix 2**

### **Arterial Ischaemic Stroke: Clinical Checklist**

This clinical checklist is not intended to be comprehensive; it is intended to supplement the conventional history and examination process by prompting the user to ask about or look for clinical factors which are potentially relevant to a child presenting with acute AIS. The aspects of the neurological examination which have been specifically highlighted are partly derived from items used in adult stroke severity scales but are not intended to replace more comprehensive clinical evaluation. If it is not possible to assess any of these parameters it is important to record this and the reasons for this (e.g. child was uncooperative or test was developmentally inappropriate)

#### **History**

- Time of onset of symptoms
- Prior events e.g. TIA
- Seizures
- Prior trauma to the head or neck
- Chickenpox? If so, when?
- History of recent infection
- Head or neck pain?
- Past medical history (incl. history of thrombosis)
- Family history (incl. thrombosis, miscarriage)
- Medication (& recreational drugs)
- Development

#### **Examination**

- Blood pressure
- Peripheral pulses (check for radio-femoral delay)
- Oxygen saturation
- Temperature
- Neurocutaneous signs
- Cardiovascular examination

Conscious level (use modified Glasgow Coma Scale)

**Neurological examination to include evaluation for:**

Cranial nerve function including:

Eye movements (?gaze palsy)

Visual fields

Facial palsy

? Horner's syndrome

Motor deficit (if present specify location and severity using MRC scale)

Ataxia (if present specify location e.g. truncal or limb)

Sensory dysfunction (if present specify location)

Communication (including expressive and receptive language and articulation)

Swallowing

Neglect (if present specify location)

**Imaging**

Brain MRI\*

MRA circle of Willis\*

MRA neck \*

\*These are all included when you request an acute "stroke protocol" MRI at GOSH

Transthoracic echocardiogram

**Blood tests**

Full blood count

Urea& electrolytes, liver function tests

ESR

CRP

Haemoglobin electrophoresis if black or Mediterranean ethnicity

Clotting profile

Thrombophilia screen (*at GOSH this will include protein C, protein S, antithrombin, plasminogen, DRVVT, Exner, APC resistance ratio, FVL, MTHFR and PT20210 gene mutations*)

Anticardiolipin antibodies

Plasma amino acids

Total plasma homocysteine

Plasma lactate

Ammonia

Cholesterol and triglycerides (random)

Plasma urate

Transferrin electrophoresis

Lp(a) (*please request on downtime form as lipoprotein (a), not available on PIMS, min. 1ml clotted blood*)

Alpha galactosidase A (to exclude Fabry disease)

Serum globotriaosylceramide (to exclude Fabry disease)

Serology: mycoplasma, VZV, borellia

### **Urine**

Organic acids

Urinary globotriaosylceramide (to exclude Fabry disease)

### **CSF**

Lumbar puncture is not routinely indicated and the need for this should be reviewed on a case-by-case basis. If a lumbar puncture is undertaken it is helpful to request CSF VZV PCR and antibodies and CSF lactate.

## Appendix 3

### Plate plan for microparticles cells

No stain  
AnV-FITC  
AnV-PE  
AnV-PECy5  
CD42a PERCP  
FITC Isotype  
PE Isotype  
PE-CY5 Isotype  
PERCP Isotype  
CD42a PERCP/AnV-FITC  
CD42aPERCP/AnV-FITC/CD62P-PE  
CD42a PERCP/AnV-FITC/CD62E-PE  
CD42aPERCP/AnV-FITC/CD31-PE  
CD42a PERCP/AnV-FITC/CD106-PE  
CD42aPERCP/AnV-FITC/aCD11b-PE  
CD42aPERCP/AnV-FITC/CD54-PE  
TF-FITC/AnV-PE/CD14 Pe-Cy5  
TF-FITC/AnV-PECy5/CD162 PE

### Plate plan for endothelial progenitor

No stain  
PE-Isotype  
PE-CY5 Isotype  
FITC-Isotype  
CD34-PERCP  
CD133-PE  
KDR- FITC  
CD144-FITC  
CD133/CD34/KDR  
CD133/CD34/CD144

## Appendix 4

### Flow cytometry instrument settings for microparticles

Detector	Voltage	Amp gain	Mode	Threshold	Compensation
Forward scatter	E01	5.57	Logarithmic	50	FL1-1.5%FL2 FL2-14 % FL1 FL2-0%FL3 FL3-18.1% FL2
Side scatter	364	1.00	Logarithmic	52	
FL1 (FITC)	690	1.00	Logarithmic	50	
FL2 (PE)	721	1.00	Logarithmic	52	
FL3 (CYC)	750	1.00	Logarithmic	52	

### Flow cytometry instrument settings for endothelial progenitor cells

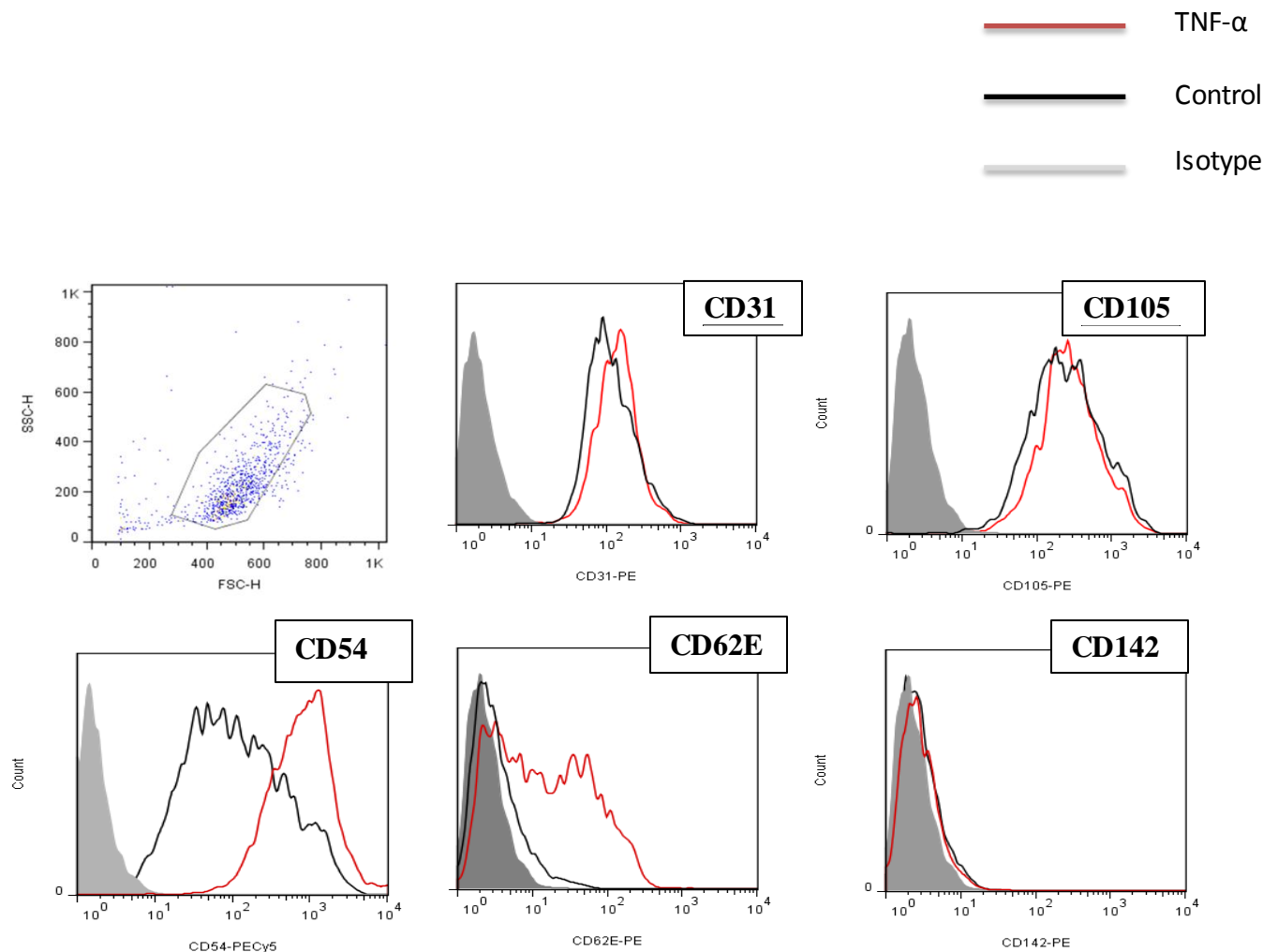
Detector	Voltage	Amp gain	Mode	Threshold	Compensation
Forward scatter	E00	1	Linear	50	FL1-0.5%FL2 FL2-19% FL1 FL2-10%FL3 FL3-17%FL2
Side scatter	480	1	Linear	50	
FL1 (FITC)	547	1	Logarithmic	50	
FL2 (PE)	627	1	Logarithmic	50	
FL3 (CYC)	437	1	Logarithmic	50	



## Appendix 5

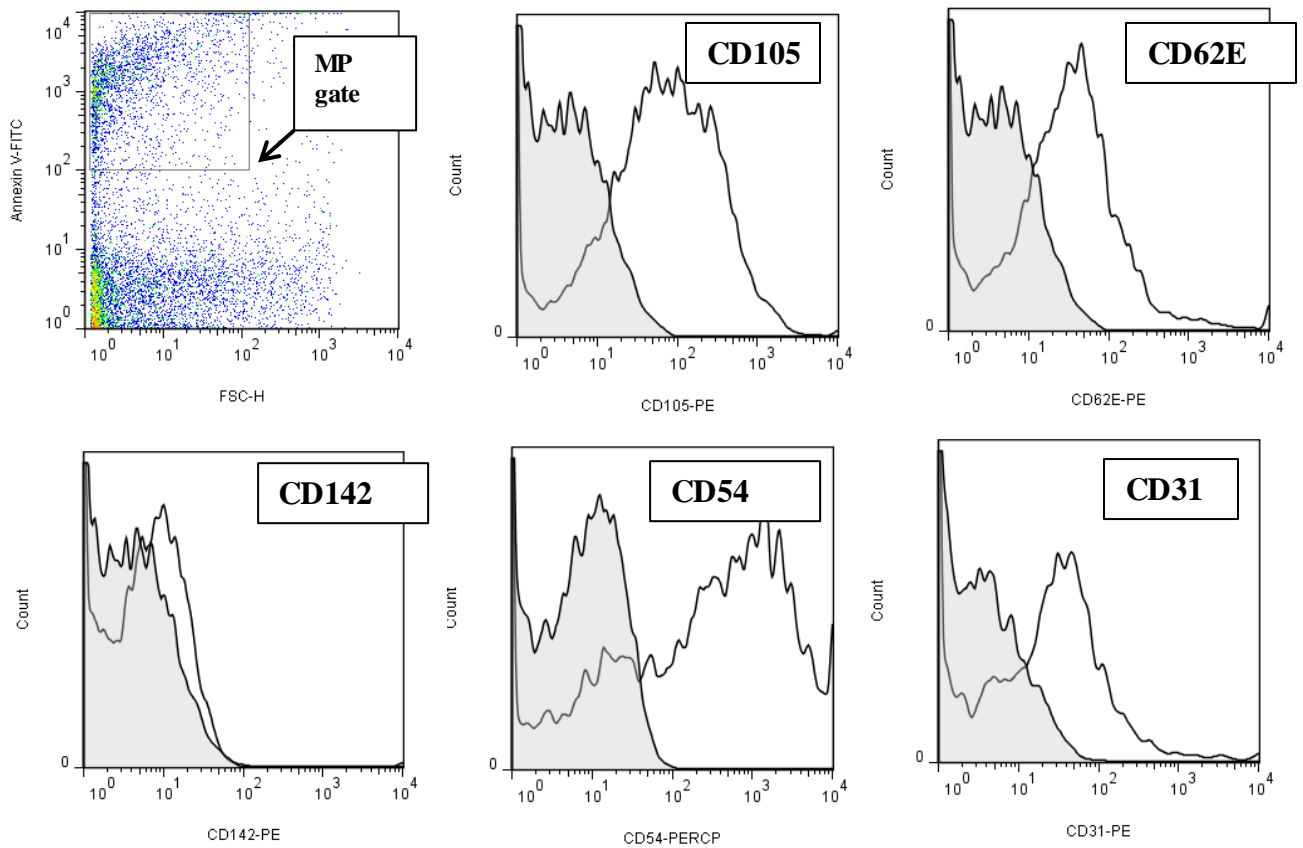
### Human umbilical endothelial cells (HUVEC)

Human umbilical endothelial cells commercially available (Promo cell) were stimulated with 100 ng/ml of TNF- $\alpha$  for 24 hours. The surface expression of a number of endothelial surface markers and tissue factor (CD142) for stimulated and unstimulated (control) HUVEC using flow cytometry are shown below.



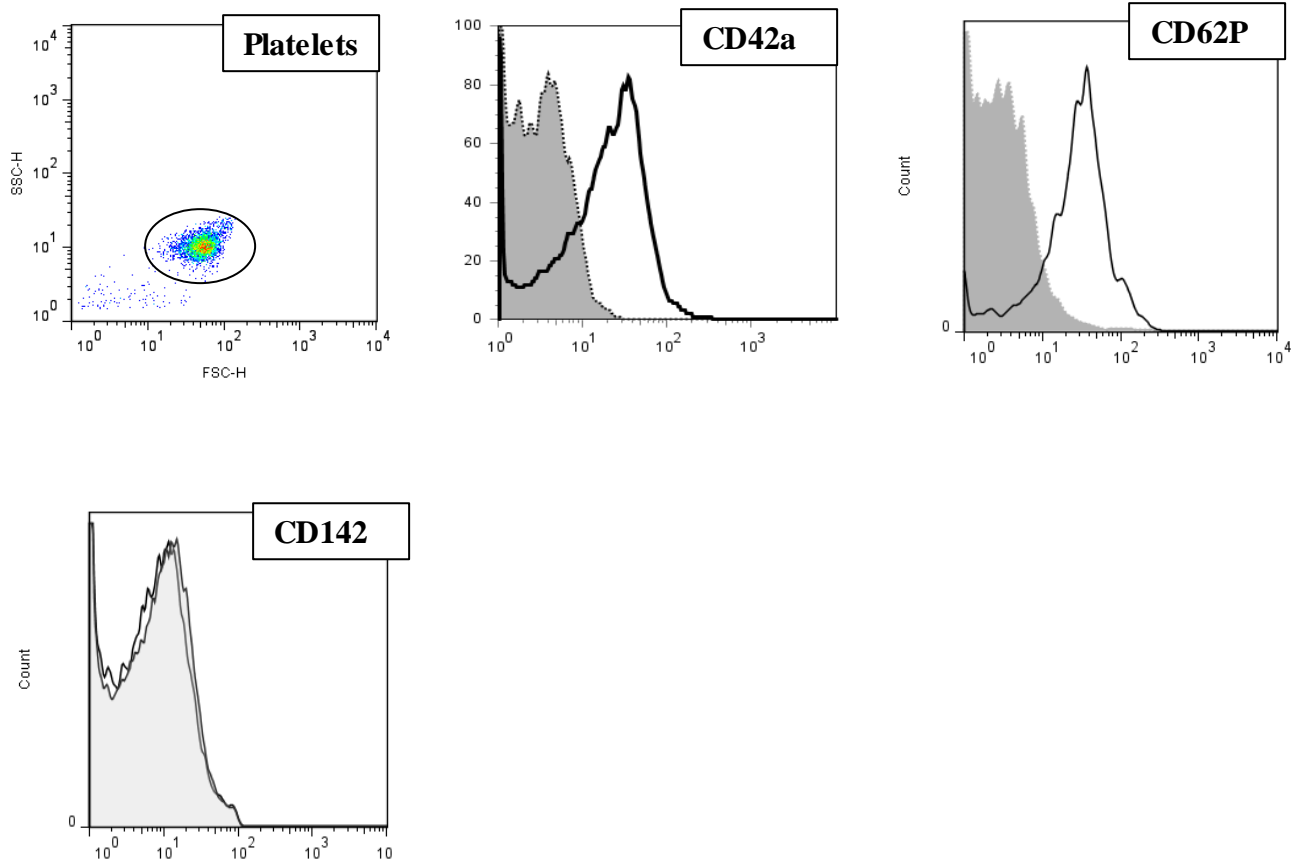
## Endothelial derived microparticles (EMP)

Endothelial derived microparticles (EMP) harvested from supernatants of human umbilical endothelial cells (HUVEC) stimulated with 100 ng/ml of TNF- $\alpha$  for 24 hours were stained for a variety of endothelial surface markers and tissue factor (CD142) –flow cytometric plots are shown below.



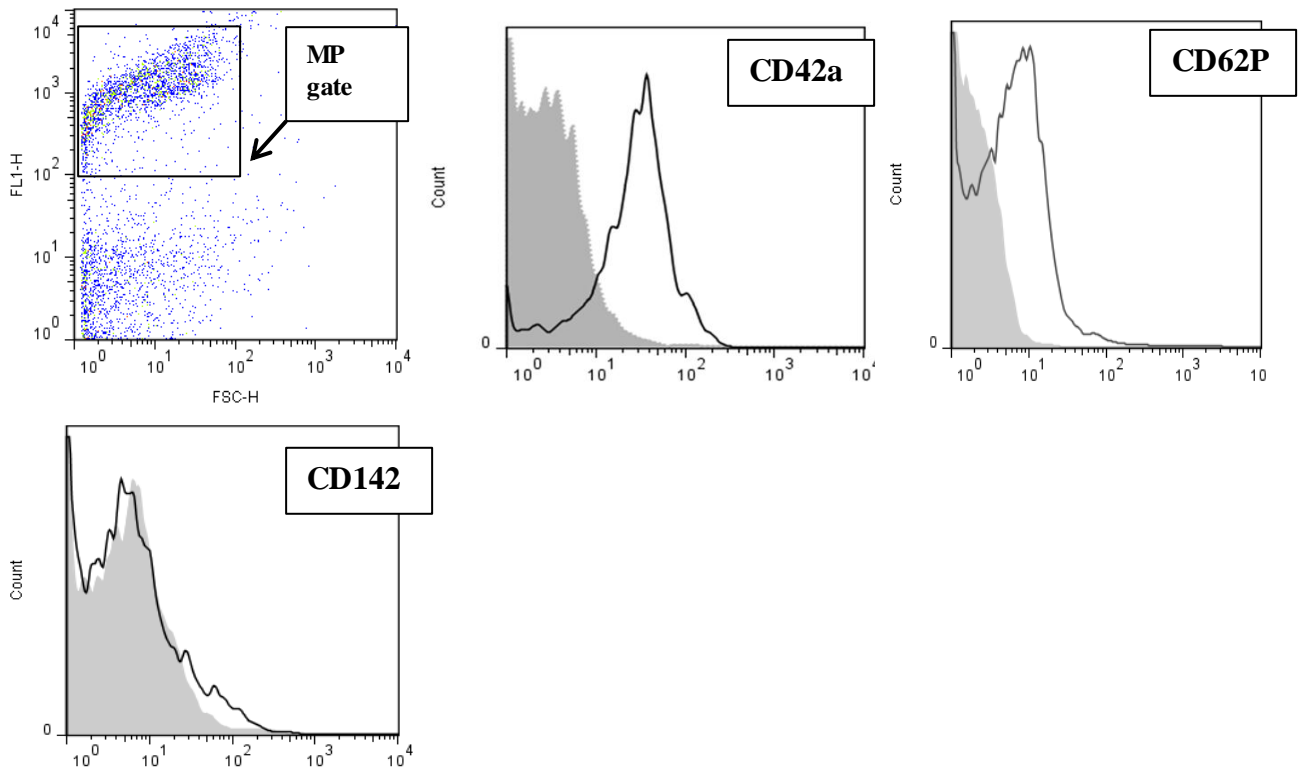
## Platelets

Platelets were isolated from human peripheral blood donated by adult volunteers and stimulated with a number of stimuli (including calcium ionophore 10 nM and ADP 5  $\mu$ M for 1hour). The surface expression of platelet constitutively expressed (CD42a) and activation markers (CD62P) as well as CD142 (tissue factor) as assessed by flow cytometry is shown below.



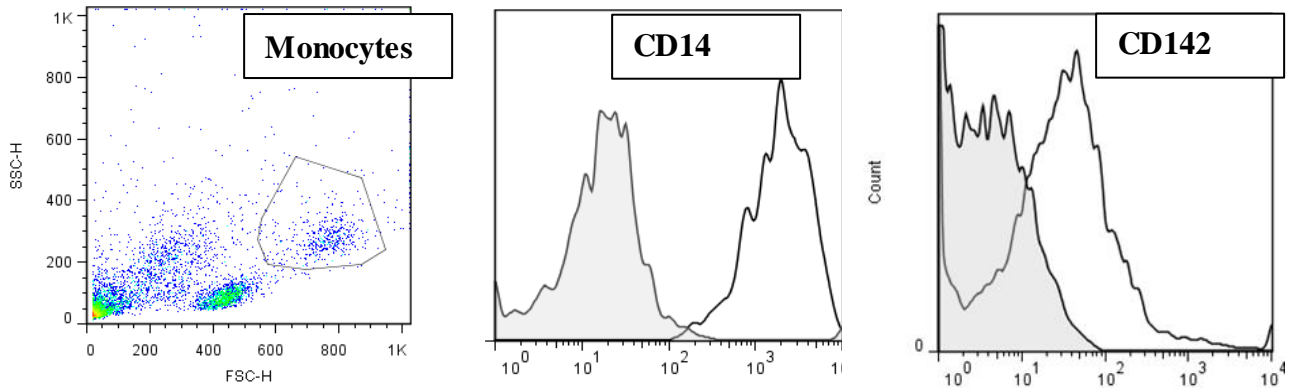
## Platelet derived microparticles

Platelet derived microparticles were harvested from the supernatants of stimulated platelets (with calcium ionophore 10 nM or ADP 5  $\mu$ M for 1hour) and stained with CD42a, CD62P and CD142 (tissue factor)-flow cytometric plots are shown below.



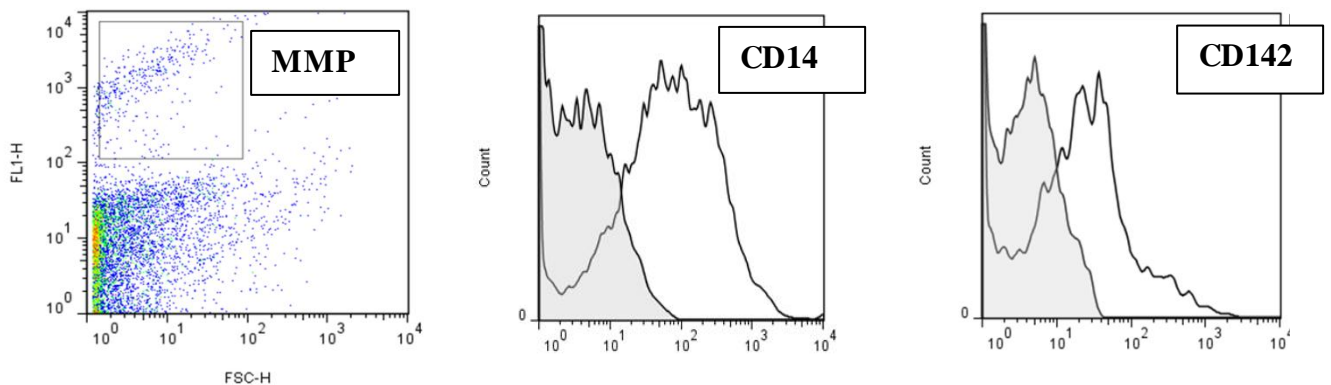
## Monocytes

Human monocytes were isolated from peripheral blood donated by adult healthy volunteers and stimulated with LPS (5  $\mu\text{g}/\text{ml}$  for 4 hours). Surface expression of CD14 and tissue factor (CD142) were assessed with flow cytometry and are shown below.



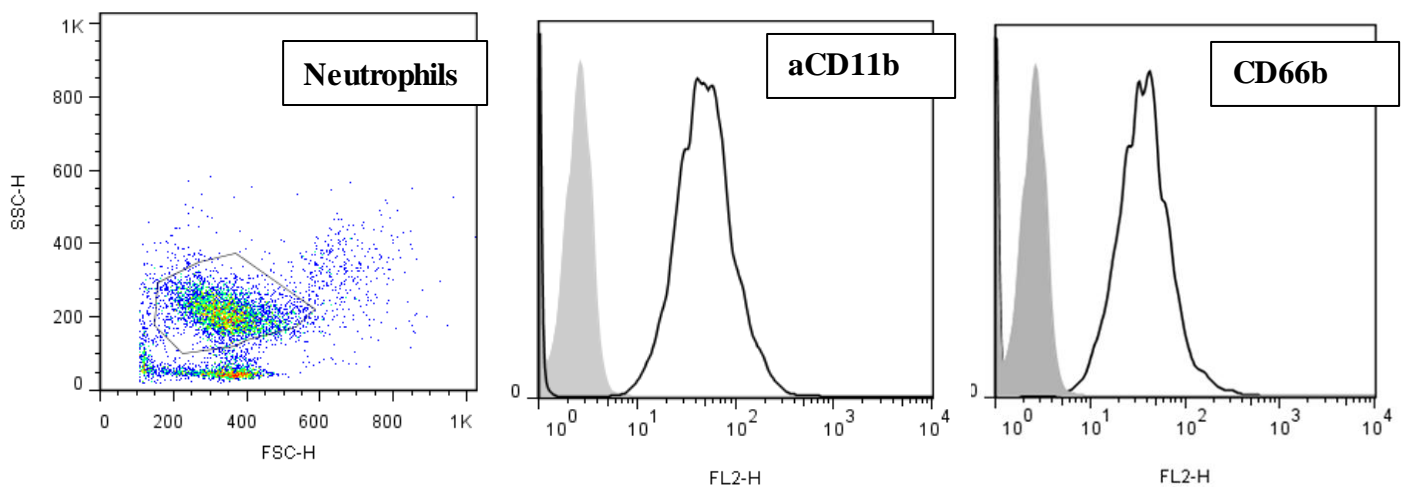
## Monocyte MPs (MMP)

Monocyte derive microparticles (MMP) were isolated from the supernatants of human monocytes stimulated with LPS (5  $\mu\text{g}/\text{ml}$  for 4 hours) and stained with CD14 and tissue factor-flow cytometric plots are shown below.



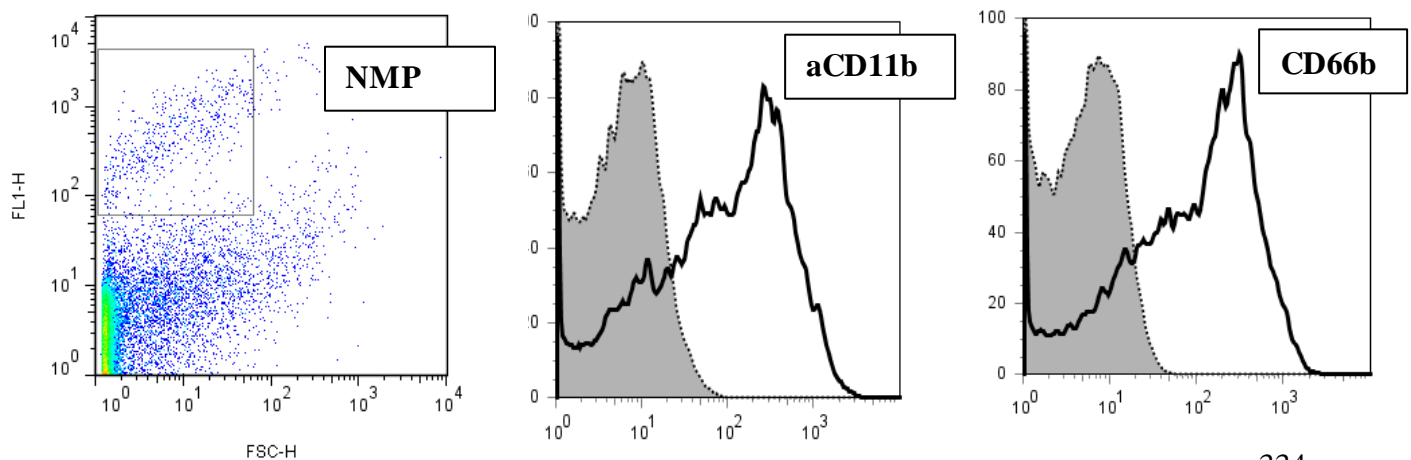
## Neutrophils

Neutrophils isolated from human blood from adult volunteers and stimulated with fmlp (10  $\mu$ g/ml for 2 hours) were stained with constitutively expressed or activation surface markers and assessed with flow cytometry (shown below).



## Neutrophil microparticles (NMP)

Neutrophil microparticles were isolated from the supernatants of human neutrophils stimulated with fmlp (10  $\mu$ g/ml for 2 hours) were stained for aCD11b and CD66b-flow cytometric plots are shown below.



## Appendix 6

### The Birmingham Vasculitis Activity Score (BVAS) (Luqmani, Bacon and others, 1994)

Tick box only if abnormality is newly present or worsening within the previous 4 weeks and ascribable to vasculitis

1. SYSTEMIC	3 (maximum total)
none	0
malaise	1
myalgia	1
arthralgia/arthritis	1
fever (<38.5°C)	1
fever (>38.5°C)	2
wt loss (1-2 kg) within past month	2
wt loss (>2 kg) within past month	3
2. CUTANEOUS	6 (maximum total)
none	0
infarct	2
purpura	2
other skin vasculitis	2
ulcer	4
gangrene	6
multiple digit gangrene	6
3. MUCOUS MEMBRANES/EYES	6 (maximum total)
none	0
mouth ulcers	1
genital ulcers	1
conjunctivitis	1
episcleritis	2
uveitis	6
retinal exudates	6
retinal haemorrhage	6
4. ENT	6 (maximum total)
nil	0
nasal discharge/obstruction	2
sinusitis	2
epistaxis	4
crusting	4
aural discharge	4
otitis media	4
new deafness	6
hoarseness/laryngitis	2
subglottic involvement	6

5. CHEST	6 (maximum total)
none	0
dyspnoea or wheeze	2
nodules or fibrosis	2
pleural effusion/pleurisy	4
infiltrate	4
haemoptysis/haemorrhage	4
massive haemoptysis	6
6. CARDIOVASCULAR	6 (maximum total)
none	0
bruits	2
new loss of pulses	4
aortic incompetence	4
pericarditis	4
new myocardial infarct	6
CCF/cardiomyopathy	6
7. ABDOMINAL	9 (maximum total)
none	0
abdominal pain	3
bloody diarrhoea	6
gall bladder perforation	9
gut infarction	9
pancreatitis	9
8. RENAL	12 (maximum total)
none	0
hypertension (diastolic >90)	4
proteinuria (>1 + or >0.2 g/24 h)	4
haematuria (> 1 + or >10 rbc/ml)	8
creatinine 125-249 $\mu\text{mol/l}$	8
creatinine 250-499 $\mu\text{mol/l}$	10
creatinine >500 $\mu\text{mol/l}$	12
rise in creatinine >10%	12
9. NERVOUS SYSTEM	9 (maximum total)
none	0
organic confusion/dementia	3
seizures (not hypertensive)	9
stroke	9
cord lesion	9
peripheral neuropathy	6
motor mononeuritis multiplex	9
MAXIMUM SCORE	63

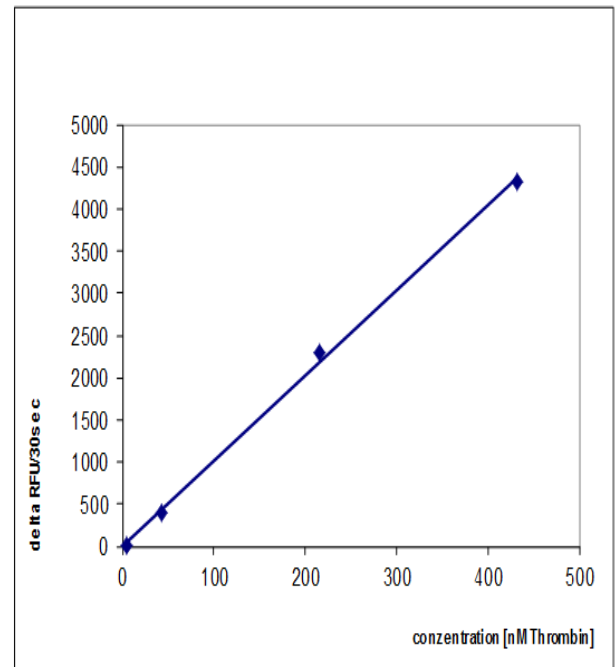


## Appendix 7

### Thrombin generation assay calibration curve

dilution	nM Thrombin	delta RFU/30 sec (W1)	OK	delta RFU/30 sec (W2)	OK	AV calculated
1: 2	432.5	4328.7		4328.7		<b>4328.7</b>
1: 4	216.25	2286.4		2286.4		<b>2286.4</b>
1: 20	43.25	396.1		396.1		<b>396.1</b>
1: 200	4.325	19.4		19.4		<b>19.4</b>

k	10.131
d	-5.9761
regression	0.9994



## **9 Publications from this thesis:**

**Eleftheriou D**, Ganesan V, Hong Y, Klein NJ, Brogan PA. Endothelial injury in childhood stroke with cerebral arteriopathy: a cross sectional study. *Neurology, In press.*

**Eleftheriou D**, Hong Y, Klein NJ, Brogan PA. Thromboembolic disease in systemic vasculitis is associated with an enhanced microparticle mediated thrombin generation. *J Thromb Haemost.* 2011;9:1864-7.

**Eleftheriou D**, Cox T, Saunders D, Klein NJ, Brogan PA, Ganesan V. Investigation of childhood central nervous system vasculitis: magnetic resonance angiography versus catheter angiography. *Dev Med Child Neurol.* 2010;52:863-7.

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**Eleftheriou D**, Ganesan V. Treatment strategies for childhood stroke. *Expert Opin Pharmacother.* 2008; 9:2955-67

**The following papers are from studies not discussed in this thesis that were carried out during the period of study and have included techniques described herein.**

Hong Y, **Eleftheriou D**, Hussain AA, Price-Kuehne FE, Savage CO, Jayne D, Little MA, Salama AD, Klein NJ, Brogan PA. Anti-neutrophil cytoplasmic antibodies stimulate release of neutrophil microparticles. *J Am Soc Nephrol.* 2012; 23:49-62

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Clarke LA, Shah V, Arrigoni F, **Eleftheriou D**, Hong Y, Halcox J, Klein N, Brogan PA. Quantitative detection of circulating endothelial cells in vasculitis: comparison of flow cytometry and immunomagnetic bead extraction. *J Thromb Haemost.* 2008; 6:1025-32.

**Papers submitted and pending peer review:**

**Eleftheriou D**, Ganesan V, Hong Y, Klein NJ, Brogan PA. Endothelial repair in childhood arterial ischaemic stroke. *Stroke Journal*, submitted.

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