Characterisation of Turner Syndrome with focus on the pathogenesis of diabetes mellitus

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Declaration

I, Antoinette Cameron-Pimblett, 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 the thesis.

Several publications have been produced as a result of this work. Published content has been stated in each of the relevant sections and chapters. Publications have also been included adhering to University College London regulations.

Publications

1. The Turner syndrome life course project: Karyotype-phenotype analyses across the lifespan. Cameron-Pimblett A, La Rosa C, King TFJ, Davies MC, Conway GS. *Clinical endocrinology.* 2017; 87:532-538.

2. Effects of estrogen therapies on outcomes in Turner Syndrome: assessment of induction of puberty and adult estrogen use. Cameron-Pimblett A, Davies MC, Burt E, et al. *The Journal of clinical endocrinology and metabolism.* 2019.

Signed,

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21/05/2020

Acknowledgements

Abstract

Introduction

Turner Syndrome (TS) is a complex condition, affecting every system in the body (Elsheikh, Conway, & Wass, 1999; Gravholt et al., 2017). Caused by a complete or partial lack of one X chromosome TS is associated with a variety of morbidities such as diabetes mellitus (DM).

A literature review found that DM is poorly characterised in TS and DM risk is reported to be higher than that of females in the general population (Gravholt, Juul, Naeraa, & Hansen, 1998). The results from glucose homeostasis reports have been conflicting reporting both insulin deficiency (V. K. Bakalov et al., 2004) and resistance (Salgin et al., 2006) to be the mechanism behind pathogenesis as well as a possible association with the isochromosome karyotype (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008). Furthermore, those with established DM have often been excluded from analysis and therefore remain largely unexplored.

The overall aim my doctoral research was to identify the factors involved in TS-associated DM using a multipronged approach.

Methodologies

A series of methodologies were implemented to address the characterisation of the DM phenotype experienced by women with TS.

Studies 1 & 2

Statistical characterisation of adult health parameter data derived from the Turner Syndrome Life Course Project against; karyotype, paediatric treatments and long-term oestrogen use.

Study 3

A prospective study of the characterisation of glucose homeostasis and DM-risk factors in affected and women with an unknown DM status.

Study 4

Pilot array study of T2DM-associated SNP to assess if there was an over-represented in those with Impaired Glucose Tolerance or DM.

Conclusions

TS-associated DM was found to have a unique profile that has features of both T2DM and LADA such as genetic influences, insulin resistance and a degree of autoimmunity.

Impact Statement

There are approximately 15,000 women living with TS in the UK today (Nielsen & Wohlert, 1991). Health and social issues surrounding TS are complex and require input from; endocrinology, gynaecology, cardiology and psychology (Gravholt et al., 2017).

TS-associated comorbidities have been widely researched (Schoemaker et al., 2008; Stochholm, Juul, Juel, Naeraa, & Gravholt, 2006). However, studies often produce conflicting results due to number of confounding issues such as small cohorts and varying methodologies.

The University College London Hospitals has one of the oldest dedicated adult clinical services for women with TS in the UK and is among the largest in the world. In 2014, the Turner Syndrome Life Course Project (TLCP) was established to document medical and psychosocial outcomes for women with TS.

The impact of the TLCP work has already been measurable. The principle publication, *The Turner syndrome life course project: Karyotype-phenotype analyses across the lifespan*, was widely received and was reviewed favourably by *Nature Endocrine Reviews*. As a reflection of the publications wider reach, I have been contacted by physicians globally regarding the clinical impact of the research. Whilst the second publication data regarding puberty and long-term oestrogen use has been presented both nationally and internationally. Furthermore, a new clinical need for a large-scale diabetes screening programme has been identified.

The TLCP data has been used in several other successful projects which have led to publications on various TS related issues (Berglund, Burt, Cameron-Pimblett, Davies, & Conway, 2019; Burt et al., 2019; Cardona Attard et al., 2019; Talaulikar, Conway, Pimblett, & Davies, 2019). Table 1 surmises my personal achievements since the initiation of my doctoral work.

In academic world, the studies completed as part of my doctoral work have provided a jumping-off point for new research at UCLH and led to a new collaboration with the Institute of Child Health. Collectively, the results have advanced our thinking about the future of genomic research in TS.

Table 1. Summary of key conference events and publications to date.

2016	Oral communication & poster presentation, Society for Endocrinology UK
2017	Oral communication, Endocrine Society USA
	First publication, Clinical Endocrinology
	Highlight piece in Nature Endocrine Reviews
	Oral communication, Women's Health University College London
	Oral communication & poster presentation, Society for Endocrinology, UK
	Oral communication, British Society of Paediatric Endocrinology & Diabetes, UK
2018	Poster presentation, European Congress of Endocrinology, Spain
	Poster presentation, Society for Endocrinology, UK
2019	Second publication, Journal of Clinical Endocrinology and Metabolism
	Poster presentation, European Congress of Endocrinology, France

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List of Abbreviations

Area Under the Curve	**AUC**
array-Comparative Genome Hybridisation	**aCGH**
Alanine Transaminase	**ALT**
Angiotensin II Receptor	**AGTR**
Anti-Glutamic Acid-Decarboxylase 65	**GAD**
Anti-Thyroid Peroxidase	**TPO**
Alkaline Phosphatase	**AIKP**
Aortic Root Diameter	**ARD**
Aortic Size Index	**ASI**
Autoimmune Disease	**AD**
Bone Mineral Density	**BMD**
Body Surface Area	**BSA**
Diabetes mellitus	**DM**
Disorders of Reproductive Development	**DRD**
Exome Sequencing	**ES**
Fluorescence In-Situ Hybridisation	**FISH**
Gamma-Glutamyl transpeptidase	**GGT**
Genome Wide Association Study	**GWAS**
Growth Hormone	**GH**
Hardy-Weinberg Equilibrium	**HWE**
Homeostasis Model Assessment	**HOMA**
Glucose Tolerance Test	**GTT**
Health Research Authority	**HRA**
High Density Lipoprotein	**HDL**
Islet Tyrosine Phosphatase 2	**IA-2**
Impaired Glucose Homeostasis	**IGH**
Impaired Glucose Tolerance	**IGT**
Insulin Receptor Substrate 4	**INSR4**
Insulin Resistance	**IR**
Insulin Sensitivity	**IS**
Integrated Research Application System	**IRAS**
Interquartile ranges	**IQR**
Polyendocrinopathy and Enteropathy X- Linked Syndrome	**IPEX**
Latent Autoimmune Diabetes in Adults	**LADA**
Low Density Lipoprotein	**LDL**
Major Histocompatibility Complex	**MHC**
Maturity Onset- Diabetes	**MODY**

Minor Allele Frequency	**MAF**
National Health Service	**NHS**
Next Generation Sequencing	**NGS**
Non-Alcoholic Fatty Liver Disease	**NAFLD**
Normal Glucose Tolerance	**NGT**
Odds Ratio	**OR**
Oestrogen Replacement Therapy	**ORT**
Oral Glucose Tolerance Test	**OGTT**
Oral Contraceptive Pill	**OCP**
Oral Oestrogens	**OE**
Primary Ovarian Insufficiency	**POI**
Pseudoautosomal Region	**PAR**
Quantitative Polymerase Chain Reaction	**qPCR**
Resting Energy Expenditure	**REE**
Reproductive Life Course Project	**RLCP**
Receiver Operator Curve	**ROC**
Single Nucleotide Polymorphism	**SNP**
T-regulatory cells	**Tregs**
Transdermal Oestrogens	**TE**
Turner Syndrome	**TS**
Turner Syndrome Life Course Project	**TLCP**
Turner Syndrome Support Society	**TSSS**
Type 1 Diabetes mellitus	**T1DM**
Type 2 Diabetes mellitus	**T2DM**
University College London Hospitals	**UCLH**
Maximal Aerobic Capacity	**VO2max**
Zinc Transporter 8	**ZnT8**

Chapter I

Literature review of Turner Syndrome, diabetes mellitus, autoimmunity and the application of gene technologies

Introduction

Turner Syndrome (TS) after endocrinologist Dr Henry Turner, and sometimes referred to as Ullrich-Turner Syndrome, was first described in a series of women with achondroplasia, amenorrhea and a lack of secondary sex characteristics (Turner, 1938; Ullrich, 1930). Today, TS is understood to be one of the most common chromosomal disorders affecting 1 in every 2,500 female live births and is caused by a partial or complete lack of one X chromosome (Nielsen & Wohlert, 1991). TS is associated with a variety of comorbidities which increase in prevalence with age (Mortensen et al., 2009).

The following is a review of TS, with a focus on autoimmunity and diabetes mellitus (DM) as well as the application of gene technologies for the advancement of understanding disease pathogenesis in TS.

Biological causes of Turner Syndrome

TS is caused by either X chromosome lag or a non-disjunction event in meiosis, in either anaphase as chromosome or chromatids of the X chromosome fail to separate to opposite poles of the cell (Zhong & Layman, 2012). On fertilisation, the resulting zygote will have a partial or complete lack of one X chromosome. In most instances, the retained chromosome is maternally derived (Hamelin, Anglin, Quigley, & Deal, 2006).

Like Down's Syndrome (Trisomy 21), also caused by a non-disjunction event, the likelihood of TS conception increases with maternal age. Hagman (2010) estimated that women aged 40+ years delivered 3.2% girls with TS compared to 1.2% of the general population (Hagman et al., 2013).

Menasha (2005) observed products of conception in order to estimate chromosomal abnormality frequency in spontaneous abortions. Menesha et al., found that 99% of products of conception and later-stage foetuses with TS spontaneously abort during the first trimester, the surviving 1% will have TS in life (Menasha, Levy, Hirschhorn, & Kardon, 2005). It has been hypothesised that the 1% born with TS must have some form of mosaicism to survive to term (Hook & Warburton, 2014). Traditional karyotyping is limited to blood lymphocytes, therefore excluding other tissues which may be 46,XX or a higher percentage of 46,XX cells. Referred to as cryptic mosaicism, Hook et al., hypothesised it may contribute towards the phenotype spectrum in TS (Hook & Warburton, 2014). It is assumed at the ovarian tissue level of those girls and women who retained regular menstrual cycles will be 46,XX.

Phenotype spectrum of Turner Syndrome

The overall phenotype of TS is variable. Contributing towards the phenotype spectrum are the many associated karyotypes, mosaicism and the potential for cryptic mosaicism. The most commonly TS-associated karyotype TS is 45,X and it is generally is accepted that those with 45,X have a more severe phenotype due to the global haploinsufficiency (Schoemaker et al., 2008; Stochholm et al., 2006; Sybert & McCauley, 2004) (see *Genetics of Turner Syndrome*).

Another factor contributing towards the phenotype spectrum is which parental X chromosome is inherited. Chromosomes carry specific patterns of methylation which alter gene expression. The inherited pattern described is referred to as genomic imprinting and is implicated in genomic disorders such as Prada-Willi Syndrome (Butler, 2009). Several studies have shown differences in methylation patterns of cells from TS subjects (Rajpathak & Deobagkar, 2014; Trolle et al., 2016) (*see Turner Syndrome-associated diabetes mellitus: evidence for genetics for further information*).

Previous studies have reported the maternal X is retained in 60 - 80% of TS cases (Sagi et al., 2007). Jacobs et al., reported spontaneously aborted foetuses had a higher prevalence of the paternally derived X chromosome (Jacobs, Hassold, Harvey, & May, 1989). A 2007 study observed 83 patients with TS and their parents to determine the origin of the remaining X and whether or not there was

a correlation with certain features of TS (Sagi et al., 2007). Participants had either 45,X or an isochromosome. Eighty-three per cent of those with the 45,X karyotype retained their maternal X chromosome compared to just 36% of those with an isochromosome. Sagi et al., found kidney malformations, increased BMI and altered cholesterol profiles were associated with those who retained the maternal X. Whilst ocular abnormalities and higher academic achievements were associated with paternal X retention. The authors concluded the short (p) arm of the X may carry specific imprinting patterns which may influence phenotype (Sagi et al., 2007). A previous report in 1997, by Skuse et al. reported similar levels of the retained maternal X chromosome and further reported that those that retained the paternal X chromosome had higher levels of executive function, improved verbal and social skills compared to their maternal X counterparts (Skuse et al., 1997). Overall, while various attributes of TS have been associated with the parental origin of the X chromosome, none have been replicated consistently to make a robust conclusion of the influence of this genetic variation.

Genetics of Turner Syndrome

TS-associated karyotypes can be divided into two subgroups; aneuploidy and structural alterations (Lebenthal et al., 2018). The aneuploidy group consist of monosomy 45,X and various forms of mosaicism i.e. 45,X/46,XX and 45,X/46,XY. The structural alterations include isochromosome X, ring X and partial X deletions. Both subgroups can exist alongside a karyotype normal cell line such as 45,X/46,XX and are described as mosaic. Rarer karyotypes include chromosomal translocations and X triploidy mosaics (Oliveira et al., 2009; Sybert & McCauley, 2004; Yesilkaya et al., 2015).

Changes to the chromosomal structure like in the isochromosome, ring chromosome and X deletions are of particular interest as they allow for breakpoint mapping. Breakpoint mapping interrogates chromosome breakpoints alongside the presence or absence of phenotypes to discover genotype and phenotype correlations. Breakpoint mapping has led to the identification of; Xp11.4 as the lymphedema critical region, Xp22.3 as responsible for the neurocognitive phenotype and Xq13.3- q27 for normal ovarian function in TS (Boucher, Sargent, Ogata, & Affara, 2001; Rizzolio et al., 2006; Therman, Laxova, & Susman, 1990; A. R. Zinn et al., 2007).

Breakpoint mapping has yet to be been used in other structural karyotypes like the isochromosome or ring chromosome. In the case of the isochromosome, a common breakpoint at q10 results in a U-shaped strand exchange which causes a partial or complete loss of the short arm (p) and a triploidy of the long arm (q) (Harel, Pehlivan, Caskey, & Lupski, 2015).

Similarly, the ring chromosome, is the result of multiple breakage-fusion events at the telomeric or distal portions of both chromosome arms creating its ring shape causing; distal and interstitial deletions or even duplications (Guilherme et al., 2013; Hu, Chai, Shu, & Li, 2018). A common breakpoint has not been identified in the ring chromosome. The ring chromosome occurs at a lower prevalence compared to other karyotype groups making genotype-phenotype correlations harder to study and has often led to their exclusion from previous research as a karyotype group (Al Alwan, 2014; V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008; Schoemaker et al., 2008; Stochholm et al., 2006; Yesilkaya et al., 2015). Furthermore, when studying potential genotype-phenotype correlations in the ring chromosome there is a further layer of complexity. In 46,XX cells, a long-noncoding RNA is activated by the *XIST* region to deactivate the X chromosome on which it is expressed randomly during the first 4 weeks of embryogenesis (Galupa & Heard, 2018). If the formation of the ring chromosome results in a large deletion, it is likely that the *XIST* locus will be lost and therefore the ring chromosome will remain active within the cell (Migeon, Luo, Jani, & Jeppesen, 1994; Migeon, Luo, Stasiowski, et al., 1994). The consequence of the active X chromosome is not yet known but it could be theorised that the remaining active X could affect gene dosage (Van Dyke et al., 1992) (*see Gene Dosage and X-inactivation for further information*).

Gene dosage and X-inactivation

Gene dosage is defined as the number of copies of each gene required to make a functional protein (Bartha, di Iulio, Venter, & Telenti, 2017). Some proteins require just one of the two available copies of a gene whilst others require both copies in equal measure. Insufficient copies of a gene are referred to as haploinsufficiency. Deviations in gene number can cause malfunctioning proteins or even a complete lack of the protein in question (Bartha et al., 2017).

The X and Y chromosome were once homologous however, evolution has limited the Y chromosome to a sex-determining function. Most X/Y ancestral genes have now been lost. The result is that 46,XX females have two copies of each X gene while 46,XY males carry just one (Graves, 2015; Peeters et al., 2019). In terms of gene dosage this implies XX individuals have more genes than XY counterparts. X-inactivation is the process by which the X to Y gene ratio is mostly balanced through the silencing of one X chromosomes in females. Those genes that do share a Y homolog do however escape X-inactivation in females. Referred to as the pseudoautosomal region (PAR), PAR 1 and PAR 2 are found in the distal regions of the p and q arms of the sex chromosomes (Peeters et al., 2019). It is haploinsufficiency of the genes located within PAR that are thought to be responsible for the TS phenotype (Andrew R. Zinn & Ross, 1998).

SHOX is a well-characterised example of the above. *SHOX* is located in PAR1 and escapes X-inactivation. *SHOX* belongs to a homeobox family of transcription regulators which controls the proliferation and maturation of chrondrocytes in growth plates and controls other downstream processes (Marchini, Ogata, & Rappold, 2016). Therefore, haploinsufficiency of *SHOX* contributes towards short stature in TS (Kosho et al., 1999).

A recent complex example of haploinsufficiency of the X chromosome is *TIMP1*. *TIMP1* is an escapee of X-activation that plays a role in aortic valve, wall formation and stabilisation. A paralog of *TIMP1, is TIMP3* found on chromosome 22. The two genes share a synergistic relationship (Corbitt et al., 2018). Corbitt et al., (2018) demonstrated by exome sequencing (ES), that when one copy of *TIMP1* is lost like in TS and occurs in tandem with a deleterious variant in *TIMP3* there is an increase in bicuspid valve and aortopathy (Corbitt et al., 2018). The theory of acquiring 'second hits' has also been described by Bakalov et al., (2008) and could explain the increased prevalence common disease such as diabetes in TS (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008) (*see Turner Syndrome-associated Diabetes mellitus: evidence for genetics for more information*).

It has been hypothesised that structural karyotypes may lead to phenotype variation and disease pathogenesis through alterations in gene dosage. Bakalov (2008) found excess transcripts not only of X-linked genes but other autosomal chromosomes in those with the isochromosome karyotype and believed to be

associated to DM pathogenesis (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008) (*see Turner Syndrome-associated diabetes mellitus: evidence for genetics for more information*).

Karyotype-phenotype correlations

There has been much research into genotype-phenotype correlations in TS (Al Alwan, 2014; V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008; Schoemaker et al., 2008; Stochholm et al., 2006; Yesilkaya et al., 2015). Clinically, the 45,X group presents on the severe end of the phenotype spectrum with the highest frequency of comorbidities as a reflection of global haploinsufficiency of X chromosome genes. Conversely the mosaic 45X/46,XX group suffer from the least comorbidities. Table 1.1 surmises all major cohort findings (Gravholt, Juul, et al., 1998; Schoemaker et al., 2008; Stochholm et al., 2006; Sybert & McCauley, 2004; Yesilkaya et al., 2015).

Karyotype-phenotype correlations have been reported in the isochromosome group, of note an excess of hearing loss and autoimmunity have been reported. Well-documented is the association between, 46,X,i(q10) and sensorineural hearing loss. In 2006 Hamelin et al., reported an excess of sensorineural hearing loss in those isochromosome subjects with a paternally derived chromosome and further found the maternal chromosome had a protective quality against sensorineural hearing loss (Hamelin et al., 2006). The isochromosome has also been linked to an excess of thyroid autoimmunity by several research groups (Elsheikh, Wass, & Conway, 2001; Grossi et al., 2013; Mortensen et al., 2009) (*see Autoimmunity of Turner Syndrome for more information*).

Relatively few studies have researched the unique phenotype experienced by those with the ring chromosome due to its low frequency within cohorts. Small ring chromosomes, which have lost the ability to deactivate, have been associated with impaired cognitive function and a greater height deficit (Migeon, Luo, Jani, et al., 1994; Migeon, Luo, Stasiowski, et al., 1994; Van Dyke et al., 1992). It is believed the inability of the ring chromosome to deactivate alters gene-dosage and leads to a severer phenotype (Migeon, Luo, Jani, et al., 1994; Migeon, Luo, Stasiowski, et al., 1994; Van Dyke et al., 1992).

Studies into the associations between TS-associated karyotypes and comorbidities are often limited. Limitations include cohorts comprised of large numbers of children and adolescences whom do not display the full TS phenotype. Smaller cohorts often mean rarer karyotypes are excluded or included within a heterogeneous group of karyotypes thus masking associations Differences in cohort analysis such as variable cut-off or defining variables also limit TS cohort studies (table 1.1) (Al Alwan, 2014; Carvalho, Lemos-Marini, Guerra-Junior, & Maciel-Guerra, 2018; Gravholt, Juul, et al., 1998; Schoemaker et al., 2008; Stochholm et al., 2006; Yesilkaya et al., 2015).

Table 1.1 summary of the major Turner Syndrome cohorts. Including mode of ascertainment and cohort size.

Cohort	Mode of Ascertainment	Cohort Size	Mean/ Median Age (Range)	Mean/ Median Age of Diagnosis
Gravholt et al.,	Registry database	594	0 - 79	-
Stochholm et al.,	Population-cytogenetics	781	-	15.1
Schoemaker et al.,	Population-death certificates	3439	-	14.5
Yesilkaya et al.,	Clinic	842	0- 18	10.4
Bakalov et al.,	Advertisement	244	35.7 (18- 67)	-
Sybert et al.,	Clinic	532	-	-

Presentation & diagnosis of Turners Syndrome

TS is a life-long condition which can be diagnosed throughout life but commonly presents during childhood through to late adolescence. A number of cases are diagnosed prenatally through foetal testing and/ or ultrasound anomalies. Ultrasound anomalies such as cystic hygromas, heart defects and increased nuchal translucency are associated with TS *in-utero* and can lead diagnosis (Conner, Longman, & Cahill, 2014; Sybert & McCauley, 2004). The incidence of aneuploidy conceptions is higher in women aged 35 or over (Hagman et al., 2010). In cases of increased maternal age, foetal genetic testing via amniocentesis and/ or chorionic villus sampling is recommended. Both techniques count foetal chromosome number and therefore may lead to a

diagnosis of TS (Conner et al., 2014). However, both procedures carry a risk of miscarriage reported between 0.5 - 1%, and although miscarriage risk may be modulated by an operator's procedure experience, it is not unusual for a woman opt-out of testing (Tabor & Alfirevic, 2010). Newer, non-invasive, testing methods such as free-cell foetal DNA, uses maternal peripheral blood to locate foetal DNA has proven useful in the diagnosis of Down's Syndrome has yet to be useful when diagnosing TS (Zhang et al., 2017). In the neonate period, the presence of congenital abnormalities such as heart and renal defects, lymphedema or ear, nose and throat issues leading to feeding issues may result in a diagnosis (Apperley et al., 2018; Carvalho et al., 2018; Yesilkaya et al., 2015). Additionally, a number of dysmorphic features are associated with TS such as a wide carrying angle, low set ears and webbed neck. These features vary in severity (Carvalho et al., 2018). A subset of dysmorphic features includes ambiguous genitalia caused by the presence of a Y chromosome fragment (*see genetics of Turner Syndrome*). The presence of ambiguous genitalia will often lead to an earlier age of diagnosis most likely at birth (Oliveira et al., 2009). In 2018, Apperley et al., examined records of 67 girls from a single centre in order to identify the mode of clinical presentation of TS. The mean age of diagnosis was 5.9 years (range pre-natal - 17.9 years). Just 10% of girls were diagnosed antenatally, whilst 16% were diagnosed in infancy, with lymphoedema and the presence of dysmorphic features respectively accounting for 27.3% and 27.3% of diagnoses respectively (Apperley et al., 2018).

Short stature is a key feature of TS and the most common route to diagnosis in childhood. The more apparent the height deficit the more likely it is for a diagnosis to be made especially if short stature is found in conjunction with stigmas such as a heart defect or ear, nose and throat issues. Apperley (2018) found short stature to be the most common presenting feature in both childhood (1 - 12 years) and adolescence (12 - 18years) (Apperley et al., 2018). A finding consistent with other less recent studies (Apperley et al., 2018; Carvalho et al., 2018; Gravholt, 2004; Yesilkaya et al., 2015).

Another common period for the TS presentation is adolescence. A deficiency of oestrogen and progesterone results in inadequate hormonal stimulus; leading to a lack of pubertal development of the breast tissue and uterus. Oestrogen deficiency results in delayed puberty and primary amenorrhoea in approximately

90% to 95% of girls with TS (Pasquino, Passeri, Pucarelli, Segni, & Municchi, 1997).

Around 5 - 10% of women with TS present during adulthood, within which there are two subgroups. In the first subgroup, there are those women who present with oligomenorrhea and secondary amenorrhoea; represents diminishing ovarian function and ovarian reserves. Therefore, woman experiencing oligomenorrhea and secondary amenorrhoea will often have symptoms of impending menopause due to oestrogen deficiency. The second subgroup, is those women that present with fertility issues. Fertility issues may range but often centre around difficulties in conception and early pregnancy retention (Doger et al., 2015). Women in either subgroup will often report normal and spontaneous puberty (Pasquino et al., 1997). For women presenting in either manner phenotype will be notably milder and they may often have a mosaic karyotype (Pasquino et al., 1997) (*see genetics of Turner Syndrome*).

One or more of the previously described features of TS will lead a clinician to request a genetic confirmation via karyotype analysis. Karyotyping is a process whereby lymphocytes are cultured from peripheral blood to a cell count of 30 or more. Chromosomes are then stained, using Giesma dye. Giesma binds to dense regions of the chromosome and creates a banded pattern referred to as G-banding. Once stained chromosomes can be examined for large scale changes or aneuploidy. For complex karyotypes involving chromosomal rearrangements and balanced translocations array-Comparative Genome Hybridisation (aCGH) may be used to interrogate breakpoints or compare the quantity of DNA when compared to a reference sample (S. J. Park et al., 2013). Similarly, Fluorescence In-Situ Hybridisation (FISH) uses probes to identify regions of importance such as those for the *SRY* and *XIST* loci (Kurnaz, Cetinkaya, Savas-Erdeve, & Aycan, 2019) (*see Genetics of Turner Syndrome for more information*).

In recent years the cost of microarrays has declined to mean the application of which in clinical diagnostics is now feasible. The advantages of microarray genotyping, as opposed to standard karyotyping, include an improved resolution which may allow for genotype-phenotype correlations to be identified (S. Prakash et al., 2014).

In 2010, Prakash et al., extended the use of microarrays to diagnose TS. One hundred and eighty-seven patients, with a previous diagnosis of TS, were genotyped using a 733,000 SNP marker array. Not only was the technology successful in identifying TS but even led to the reclassification of some individuals to a mosaic karyotype (S. Prakash et al., 2014). More modern techniques such as Next Generation Sequencing (NGS) (Rivkees, 2012) and exome sequencing (Murdock et al., 2017) have been proven to detect TS with greater accuracy for breakpoint detection in structural anomalies but are yet to become widely utilised in clinical diagnostics.

Management of Turner Syndrome & comorbidities

In 2017 new international guidelines regarding the management of TS across an individual's life course were published (Gravholt et al., 2017).TS associated comorbidities increase in prevalence during adult life (Gravholt, Juul, et al., 1998). Hanew et al., further described women with TS as having an increased risk of lifestyle-related disorders such as obesity and hypertension (Hanew et al., 2016).

Annual health surveillance is recommended to assess liver function, DM-risk, and thyroid function. For those with regular cycles or who have entered spontaneous puberty, ovarian reserve may be assessed by anti-mullerian hormone, luteinising hormone, follicle stimulating hormone and oestrogen (Gravholt et al., 2017). More recent research using the UK Biobank suggests that those with 45,X/46,XX should receive minimal clinical as they often do not experience the same health issues as women with other TS-associated karyotypes (Tuke et al., 2019).

Other surveillance includes an ECHO cardiogram to assess Aortic Root Diameter (ARD), thyroid and coeliac autoantibody screening and for bones a DEXA scan for bone mineral density assessment (BMD) which are performed at 5 yearly intervals. The scans allow for assessments of aortic dissection and osteoporosis risk (Bondy, 2008; Conway, 2002).

Treatment of Turner Syndrome

Girls with TS experience short stature as a consequence of poor response to growth hormone (GH) by tissues. To address the subsequent short stature GH is prescribed. The 2017 TS management guidelines recommend that GH treatment should commence around ages 4 - 6 years and preferably before the age of 13 (Gravholt et al., 2017).

The majority of adolescents with TS do not enter spontaneous puberty due to gonadal dysgenesis causing oestrogen deficiency (Pasquino et al., 1997). For these girls' puberty is induced and initiated around age 11 - 12 to mimic natural development (Burt et al., 2019). Doses of oestrogen are gradually increased over time, the preparation of oestrogen administration varies, with transdermal patches often preferred (Gravholt et al., 2017). Oestrogen doses are increased until adult uterus and breast size are achieved along with breakthrough bleeding (Gravholt et al., 2017). Once a breakthrough bleeding occurs, progesterone is used to initiate monthly withdrawal bleeds (Gravholt et al., 2017). After this point, Oestrogen Replacement Therapy (ORT) preparations differ and will be tailored around an individual's needs and comorbidities. ORT is required to replace absent oestrogen until the age of 50 when oestrogen is slowly withdrawn to mimic natural menopause (Gravholt et al., 2017). Whilst little data exists on the long-term effects and use of ORT in TS, more recent studying have found earlier exposure to oestrogen to be beneficial to BMD (Nguyen et al., 2018; Nguyen et al., 2017).

Treatment for those with regular menstrual cycles may differ from that of the above. Treatment of most comorbidities associated TS such as hypertension and dyslipidaemia are much the same as in the general population.

Autoimmunity in Turner Syndrome

Autoimmune Disorders (AD) are a heterogeneous group of conditions characterised by the immune system's inability to recognise self. To date, 80 different autoimmune conditions have been identified including various thyroid disease and T1DM (Gawlik et al., 2018). The prevalence of autoimmune disorders is twice as high in the general female population as opposed to male

(Gawlik et al., 2018; Jorgensen et al., 2010). Table 1.2 summarises these studies their methods and findings.

Autoimmunity in TS has been reported to occur in up to 50% of cases (Mortensen et al., 2009). Mortensen (2009) studied 107 of TS girls and women (median 36.7 years, range 6-60 years). The presence of 5 autoantibodies was tested for including; total immunoglobulin (Ig), IgA anti-gliadin and anti-transglutimase IgG, anti-thyroid peroxidase (TPO) and anti-glutamic acid-decarboxylase 65 (GAD). From 106 subjects, 58% tested positive for one or more autoantibodies. Thyroid autoimmunity was the most common 48% compared to 13% in the general population. GAD autoantibodies were found in 4/106 subjects of which 2/4 were classified as type 2 diabetics (T2DM). The remaining participants tested either TPO or coeliac disease positive. Interestingly, 75% of subjects that were GAD+ had an isochromosome. Autoantibodies were absent in the youngest members of the series aged 6-11 years. Authors concluded that autoimmune prevalence increases with age (Mortensen et al., 2009).

The largest study of AD was a retrospective study conducted between 1980-2004 using the Danish Cytogenetic Central Register. Forty-six different AD were identified in 882 women with TS (Jorgensen et al., 2010). Jorgensen et al., the risk of AD in TS was twice that of Danish women especially those which were predominately associated with males such as T1DM (Jorgensen et al., 2010). A later report by Grossi et al., tested 66 girls and women with TS (age range 1-29.8 years) for autoantibody frequency and karyotype associations. Autoantibodies tested were; TPO and thyroglobulin for thyroid, GAD and islet tyrosine phosphatase 2 (IA-2) for markers of DM, IgA, anti-gliadin and anti-transglutimase for coeliac disease. Thyroid function was also measured via TSH and T4. Thyroid autoimmunity was the most common autoimmune disorder (39.4%). Hashimoto's thyroiditis and Grave's Disease was found in 18.2% of subjects respectively. Just 2/66 subjects were reported to have positive DM-associated autoantibodies. One patient was reported to have DM-associated autoantibodies with no overt symptoms of the disease. Whilst the other was reported to have Hashimoto's Thyroiditis and T1DM. An excess of thyroid disease was associated with the isochromosome group when compared to other karyotype groups such as 45,X. The authors concluded that autoimmunity increases with age in TS (Grossi et al., 2013).

Finally, girls and women with TS not only have an increased prevalence of AD but have also had reported differences in immune cell numbers. A 2018, study of 37 girls with TS reported differing numbers of immune system cells in the TS population when compared to controls. Gwalik et al., reported lower levels of CD4 cells and CD4:8 ratio in those with TS. No other differences found between the groups, a within TS analysis found that those individuals with the isochromosome had a lower T-regulatory cells (Tregs). Tregs normal function is to maintain tolerance to self by suppressing the activation, proliferation and effectors of a variety of immune system cells. Lower levels of Tregs may suggest a lower tolerance to self and greater autoimmune risk (Gawlik et al., 2018).

Table 1.2 summary of previous research characterising autoimmune disease in girls and women with Turner Syndrome.

Author	N	Cohort Mean Age (range)	Methodology	Study Results & Conclusions
Mortensen et al., 2009	107	36.7 (6-60)	Autoantibody testing for gliadin, transglutaminase, adrenal cortex, intrinsic factor, TPO and GAD.	58% subjects were positive for one or more autoantibodies. Thyroid autoimmunity was reported in 48% and 4/106 were GAD+. T2DM was found in 2/4 of the GAD+ subjects, the other subjects had no previous DM history. TPO+ coexisted with GAD+ in one case. However, one case of T1DM did not test GAD+.
Jorgensen et al., 2010	789	15 (0-85)	Used the Danish Cytogenetic Central Register to identify women with TS who had also experienced AD	Women with TS were significantly at risk of AD such as Hashimoto's thyroiditis and T1DM. Those with 45,X and isochromosome were found to be at a 2-fold and 3-fold risk of AD of female-associated AD. Similar results, were noted for male-predominant disorders.
Grossi et al., 2013	66	N/A (1-29.8)	Autoantibody and karyotype associations.	Thyroid autoimmunity was reported as the most common disorder. 2/66 were reported to have DM associated autoantibodies. Isochromosome was associated with autoimmunity.
Gwalik et al. 2018	37	12.8 (3.4-18.2)	Girls with TS were compared to 11 healthy controls. Measurements of immunoglobulins, lymphocyte cell populations, markers of inflammation, and thyroid autoantibodies.	Girls with TS were reported to have lower levels of CD4 cells and CD4:8 ratio. The isochromosome X was further found to be associated with lower Treg cells. Changes in immune cell profiles may suggest girls with TS may be at risk of AD through a lower self-tolerance.

Diabetes mellitus in Turner Syndrome

TS has an 11-fold increased risk for T1DM and 4-fold increased risk for T2DM compared to the general population (Gravholt, Juul, et al., 1998). T1DM is characterised by   childhood onset as a result of an autoimmune response damaging liver beta-cells rendering them unable to produce insulin. The inability to produce insulin means patients with T1DM must take insulin to absorb glucose from the bloodstream. T1DM is classified as an AD and can occur in conjunction with other AD such as thyroid disease (Nederstigt et al., 2019).

In contrast, T2DM has a later onset with complex aetiology. A high fat/ sugar diet, obesity and a sedentary lifestyle all contribute towards T2DM risk. Disease pathogenesis risk can be compounded by an individual's genetics i.e. a positive family history of DM. Ethnicity also plays a role in T2DM risk in the general population with a 6-times increased risk in South Asian populations, 3-times increased risk in African and Afro-Caribbean populations (Goff, 2019). Prolonged elevation of blood glucose over time causes beta-cell damage and mreduced insulin secretion through the promotion of beta-cell apoptosis (Donath et al., 2005). The mechanism of DM in both instances is poorly understood and under characterised in TS.

In the general population, DM does show some sex bias. T1DM shows no sex bias before the age of 15. However, in European populations, as age increases as does the prevalence amongst males, with an estimated 3:2 male to female ratio (Gale & Gillespie, 2001). Transmission of T1DM, has also noted to be higher from affected father to offspring as opposed to an affected mother. It is also noted that women of reproductive age are less likely to develop T1DM also reducing the likelihood of transmission to offspring (Gale & Gillespie, 2001). T2DM is reported to affect both men and women equally. However, men have been reported to be more susceptible to risk factors such as obesity. Unlike T1DM, those affected T2DM mothers are more likely to transmit susceptibility to their offspring than affected fathers (Gale & Gillespie, 2001). In regards to TS, sex bias transmission of comorbidities such as DM is yet to be explored. But could explain a new mechanism of acquiring a 'second hit' and phenotype variation which, as previously mentioned, has been seen to be involved in the incidence of bicuspid aortic valve and aortopathy in TS (Corbitt et al., 2018).

Current classifications of DM are based on a patients age of presentation and insulin dependency. Table 1.3 shows the current classification of DM according to American Diabetes (American Diabetes, 2015). Included in the classification are the features of TS-associated DM. The American Diabetes classification fails to acknowledge how individuals with TS-associated DM often do not fit into distinct categories and share characteristics across subtypes. There are also two other subtypes which do not fall distinctly in the American Diabetes classification; DM Maturity-Onset Diabetes of the Young (MODY) and Latent Autoimmune Onset Diabetes in Adults (LADA).

MODY is a rare genetic form of diabetes defined as; onset before the age of 25, with no associated autoimmunity and sustained beta-cell function (Urakami, 2019). Six subtypes have been identified. The most common mutations are found in genes *HNFA1* and *HNFA4* (Leighton, Sainsbury, & Jones, 2017; Urakami, 2019). Each subtype an associated gene with a slightly different phenotype, however, as well as the previously mentioned characteristics most cases present as nonobese, with hyperglycaemia and positive family history (Urakami, 2019).

LADA is generally thought of as an autoimmune subtype of DM (B. Liu, Xiang, Liu, & Zhou, 2019). Similar to T1DM an autoimmune response causes beta-cell function loss and insulin resistance and in this respect, LADA can be thought of a slowly progressive insulin-dependent form of DM (Awata et al., 2017). Due to the heterogeneous nature of LADA in terms of autoimmunity, genetics and clinical features it has been suggested that LADA may bridge the gap between T1DM and T2DM displaying a diabetes continuum (B. Liu et al., 2019).

Rarer still are X-linked disorders with a DM phenotype such as Polyendocrinopathy and enteropathy X-linked Syndrome (IPEX). Children born with IPEX are described as having a severe neonatal T1DM onset and immune deregulation. A candidate gene approach led to the identification of *FOXP3. FOXP3*, Xq11.23-q13.3, is responsible for T-cell suppression and regulation of immune responses. Studies have shown that disruptions to *FOXP3* promote beta-cell apoptosis and lead to T1DM (Bacchetta, Barzaghi, & Roncarolo, 2016).

C-peptide testing can be implemented to assess beta-cell function and can help to distinguish DM subtypes (Leighton et al., 2017). C-peptide is the prohormone of insulin which assists in; insulin folding, targets specific tissue cell membranes

and initiates signalling cascades within the cell (Yosten, Maric-Bilkan, Luppi, & Wahren, 2014). Low levels of C-peptide are associated with T1DM eventual loss of beta-cell function (Davis et al., 2015; Yosten et al., 2014). C-peptide can also help in the diagnosis of MODY or LADA and in determining if insulin therapy is appropriate in the treatment of T2DM (Leighton et al., 2017).

Table 1.3 shows the key features of diabetes mellitus subtypes.

Diabetes mellitus Subtype	Features
T1DM	Autoimmune mediated through beta cell destruction Insulin deficient Childhood onset Insulin deficient Inheritance pattern
T2DM	Range of presentation from insulin resistant (IR) with some insulin deficiency to a secretory defect with IR Genetic basis affecting beta-cell function or insulin action Linked to an obese and sedentary lifestyle Adult onset Inheritance pattern
Maturity-Onset Diabetes of the Young (MODY)	Genetic Age of onset before 25 Non-autoimmune Nonobese Beta-cell function retained
Latent Onset Diabetes of Adults (LADA)	Autoimmune mediated through beta cell destruction Insulin deficient Idiopathic Associations with autoimmune thyroid disease Early adulthood onset DM- specific autoimmunity unknown Links to obesity

Previous research has yet to characterise the DM phenotype in TS. As shown in table 1.3 TS-associated DM does not easily fall into either the T1DM or T2DM category, sharing features with both subtypes. Anecdotally it is not uncommon for women with TS to be diagnosed with T1DM based on the age of DM presentation or use of insulin without DM autoantibody screening. It should be noted that within the non-diabetic population GAD autoantibodies are found in around 1.75% of the population (Sorgjerd et al., 2015).The prevalence of GAD autoantibodies increased in those with DM; 4.2% of T2DM and 73% of those with T1DM (Narendran, Estella, & Fourlanos, 2005; Zinman et al., 2004). It has been suggested that GAD autoantibody positivity is linked to the progression of LADA and reduced fasting insulin in those with T2DM (R. Turner et al., 1997; Zinman et al., 2004).

Evidence for the mechanisms of Turner Syndrome-associated diabetes mellitus

In the following section, evidence for each of the potential factors influencing TS-associated DM from previously published research. Many of the studies encompass different areas such as glucose homeostasis and the influence of oestrogen. Therefore, some studies are mentioned several times. Table 1.4 summaries these studies.

Table 1.4 summarises the major diabetes studies in Turner Syndrome. Included are the study design, cohort sizes as well as numbers of diabetic subjects, results and conclusions.

Author	Year	Cohort Size	Number of Diabetics	Cohort Mean Age (range)	Controls	Research Methodology	Study Results & Conclusions
Caprio et al.,	1991	13	N/A	Group1: 10 +/- 0.8 Group 2: 17.6 +/- 1.4	TS age-matched controls	To evaluate if IR contributes to DM-risk in TS.	Glucose metabolism was found to be impaired and accounted for by nonoxidative glucose disposal. Due to the presentation of IR in paediatric years, authors concluded IR was intrinsic to TS.
Gravholt et al.,	1998	26	N/A	33.1± 7.9	Age-matched healthy controls	Influence of differing ORT modalities on glucose homeostasis. Physical fitness was also investigated.	Insulin response was lower in TS subjects particularly in the first phase. 50% of TS subjects had IGT prior to ORT. Which increased to 78% after treatment. Despite an increase in IGT during ORT use authors were not sure if this was a true oestrogen effect. Evidence of IR was also present. Furthermore, TS subjects had significantly higher adiposity and reduced physical fitness.
Bakalov et al.,	2004	25	3	30± 9	Age-BMI matched POI controls	Investigate whether IGT and DM is independent to obesity and hypogonadism via oral and intravenous GTT in TS women.	TS subjects had an increased incidence of IGT (36%) and decreased insulin response found to be secondary to obesity and hypogonadism. 12% of TS were diagnosed with T2DM during the study. Authors concluded that the haploinsufficiency of the X chromosome predisposes women with TS to IGT and DM.
Salgin et al.,	2006	16	N/A	30.2± 8.2	Age-matched healthy controls	Assessment of insulin sensitivity in TS women.	IR in women with TS is independent to obesity and possibly due to an intrinsic defect.
Alves et al.,	2006	9	N/A	23± 4.9 (18-32)	Self-controlled	Assessment of the Influence of ORT on insulin resistance and body composition.	Changes to ORT administration does not affect insulin tolerance. Transdermal estradiol was associated with higher lean body mass.
Bakalov et al.,	2008	224	27 further 30 diagnosed	35.4± 11.2 (18- 67)	Healthy controls	Prospective OGTT and DM-associated autoantibody testing. qPCR conducted to detect gene expression differences.	Higher glycaemic and lower insulin responses in TS subjects. 30/224 diagnosed with T2DM and 23.2% of TS subjects had IGT. 14/ 113 women were GAD+. 3/14 had previous history of DM. The isochromosome was found to have increased expression of Xq transcripts which could lead to over-expression of T2DM-associated genes and GAD+ status.
Hjerrild et al.,	2011	13	N/A	33.2 ± 4.8	Healthy controls	Study of glucose homeostats, insulin sensitivity and beta-cell function.	Women with TS were similar in terms of insulin sensitivity and beta-cell function when compared to well-matched controls. However, TS subjects did show a higher prevalence of IGT despite being within a healthy weight range.
Santiago-Torres et al.,	2013	40	N/A	16.7± 1.7	N/A	Investigation into the metabolic effects of oral versus transdermal ORT	Delivery of ORT does not affect body composition or the other metabolic pathways like lipid oxidation.
Baronio et al.,	2017	104	N/A	9.1± 3.4	N/A	7-year follow-up investigation into the effects of GH on glucose and insulin	15/104 girls had IGT. Despite a normal weight, girls with TS have reduced IS. IS or glucose homeostasis did not deteriorate with the use of GH.

Ibarra-Gasparini et al.,	2018	113	14	32 (20-61)	N/A	Follow study of OGTT, DM-autoantibody testing and c-peptide.	DM in TS is characterised by a reduced first phase insulin response leading to a deficit. DM prevalence was 12.4%. 2/14 were GAD+. One subject was also IA-2+. Both subjects had an isochromosome. The use of HbAc1 in the detection of DM was found to be limited.

Turner Syndrome-associated diabetes mellitus: evidence for the role of insulin resistance and obesity

Insulin resistance can be tested in two ways; insulin resistance (IR) and insulin sensitivity (IS). There have been many reports in TS regarding IR and IS, most have centred around children and GH therapy (Baronio et al., 2017; Caprio et al., 1991).

A 1991 by Caprio et al., report measured IR indirectly using euglycemic insulin clamp to assess IS as well as the use of indirect calorimetry to measure whole body glucose and lipid oxidation. Two groups were studied. Group 1, consisted of 8 young patients (mean age 10 years) who had never received hormone therapy. Group 2, consisted of 5 older subjects (mean age 17.6 years) who had been or were on oestrogen therapy. Both groups were age matched to controls. During the euglycemic clamp, insulin-stimulated glucose metabolism decreased in both groups and was associated insulin-induced suppression of hepatic glucose production, thus suggesting that IR in TS could be an early defect that is not restricted to nonoxidative pathways on intracellular glucose metabolism (Caprio et al., 1991). A recent 7-follow up study, of similar methodology, found that GH therapy did not impact insulin sensitivity (Baronio et al., 2017). Of the 104 girls studied, 15/104 were identified as having IGT at some stage and although there was no significant change from baseline calculated HOMA-IR girls with TS were found to be relatively IR (Baronio et al., 2017).

An adult study of 16 women (mean age 30.2 years) Salgin et al., aimed to determine whether insulin resistance and adiposity were an intrinsic defect to TS or if IR was the result of body compositions differences in TS. Fasting insulin and glucose samples were taken and a hyperinsulinemia euglycemic clamp was performed to assess peripheral insulin sensitivity and Homeostasis Model Assessment (HOMA) was used to estimate fasting insulin sensitivity. Finally, body composition was measured using dual-energy X-ray absorptiometry scan. The results indicated that fasting insulin sensitivity was lower in TS subjects when compared to controls as well as whole-body IS. However, there was no association between sensitivity and abdominal fat mass in between or within either study group. In addition, researchers found that women with TS and a normal fasting glucose were more IR than controls, even when height and fat mass differences were corrected for. Therefore, low sensitivity found to be

independent to fat-free mass. Salgin et al., concluded that women with TS demonstrate an impaired peripheral and hepatic insulin sensitivity, which was statistically associated with the 45,X karyotype, which was independent to body composition (Salgin et al., 2006).

A later study by Bakalov et al., performed glucose tolerance tests alongside DM-specific autoantibody testing (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008). In a prospective study of 226 adults with TS mean age 32.7 years (range 18-67 years old) and 30 female controls with a non-significant history. Of the 224 women submitted for an OGTT, 27 had pre-existing DM. A further 30 women were diagnosed with DM during the study. Just one subject was previously diagnosed with T1DM. The total number of reported diabetics for the cohort was 25.4%. Furthermore, 52% of the cohort was diagnosed with IGT which was found to be compounded by increasing age and obesity. Insulin sensitivity was calculated from OGTT glucose and insulin both at fasting and globally for post glucose load. However, insulin sensitivity, was found to be similar to controls (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008).and was support by earlier research (Salgin et al., 2006).

Turner Syndrome-associated diabetes mellitus: evidence for insulin deficiency

As well as insulin resistance, insulin deficiency has often been described in TS (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008; V. K. Bakalov et al., 2004; Ibarra-Gasparini et al., 2018). With many reports describing a reduced first-phase insulin response in those with TS when compared to both healthy and oestrogen deficient controls (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008; Ibarra-Gasparini et al., 2018).

 In 2004, Bakalov et al., sought to establish if impaired glucose homeostasis (IGH) in TS was independent to obesity and/ or oestrogen deficiency. Twenty-five in women with TS were age and BMI matched to women with POI. Glucose levels were higher amongst those with TS, which did not match insulin secretion which was reduced especially in the first phase by 60%. Thirty-six percent of women were diagnosed with IGT and 12% were found to have T2DM after 120' minute glucose tolerance test (GTT). Researchers concluded that IGH was independent of obesity and hypogonadism was intrinsic to TS and potentially

caused by the haploinsufficiency of X chromosome genes impairing beta-cell function and creating a predisposition to IGT/DM. A later study by the same group, produced similar results also concluded that insulin deficiency was the cornerstone of DM pathogenesis in TS and found the DM phenotype to be more representative of MODY than other DM subtypes (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008) (*see evidence for the role of insulin resistance and obesity for study details*).

A small study (2011) of 13 women with TS (mean age 33.2 ± 4.8) assessed glucose homeostasis. Insulin and beta-cell function. TS subjects were compared to age, BMI and to a certain extent fat-mass matched healthy control. Unlike previous studies there was no difference between TS subjects and controls in insulin sensitivity. The authors reported that when controlled adequately, women with TS of a normal weight show IGT but normal insulin secretion and sensitivity (Hjerrild et al., 2011).

Ibarra-Gasparini (2018) also noted in those subjects that were diagnosed with DM as a result of OGTT insulin response to glucose load was found to be markedly reduced. Insulin was found to be particularly deficient during the first 60 minutes on the OGTT, in the subsequent 60 minutes insulin was found to increase. When compared to age and BMI women with POI similar results were observed. The authors concluded that the reduced first phase insulin response was caused by a defect in TS and not a result of oestrogen deficiency (Ibarra-Gasparini et al., 2018).

Turner Syndrome-associated diabetes mellitus: evidence for role of autoimmunity

There has been a little research into autoimmunity and autoantibody prevalence in TS (Gawlik et al., 2018; Grossi et al., 2013; Jorgensen et al., 2010; Mortensen et al., 2009). But just two studies to date have assessed glucose homeostasis as well as DM-associated autoantibodies (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008; Ibarra-Gasparini et al., 2018).

As part of the Bakalov et al., (2008) study autoantibodies for GAD and islet cells were tested for in 113 women (total study n= 224). None of the women tested positive for islet autoantibodies. However, 14/113 did test positive for GAD; 1/14

had T1DM, 2/14 had T2DM, 4/14 had IGT the remaining 5 women had NGT. There was a higher prevalence of GAD positive autoantibodies in the isochromosome group when compared to other karyotype groups (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008). Furthermore, there was also a global over-representation CRP, a marker of inflammation and autoimmunity, and IGF2 (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008).

The most comprehensive study of glucose homeostasis is that conducted by Ibarra- Gasparini et al,. The prospective follow-up study conducted OGTT aimed to determine how many women with TS develop IGT or DM over a 5-year period. The study included not only DM-specific autoantibodies but measured C-peptide measurements throughout the OGTT duration. Of the 113 women (mean age 32) studied during the prospective stage of the study, 14 were diagnosed with T2DM and a further 2 had previously established DM. Just 2/14 subjects tested GAD positive, one of which also tested islet-cell autoantibody positive. Coincidentally, both patients had an isochromosome. The total DM prevalence in the Italian cohort was 12.4%. IGT was found in 26% of the population. Age was found to be the most significant factor in IGT and DM-risk when compare to those who were Normal Glucose Tolerance (NGT). BMI and oestrogen use were found not to play a role in pathogenesis. Researchers found insulin secretion to be insufficient in those with TS-associated DM. The results for C-peptide measurements were not reported on publication (Ibarra-Gasparini et al., 2018).

Turner Syndrome-associated diabetes mellitus: evidence for the role of oestrogen

The majority of women with TS are oestrogen deficient and require oestrogen replacement therapy (ORT), not only this but girls and women will change their preparations over time (Gravholt et al., 2017; Pasquino et al., 1997). Gravholt et al., examined glucose and lipid metabolism in women with TS. Eight women with TS were matched to healthy controls and subjected to; intravenous and oral glucose tolerance tests (OGTT), lipid metabolism testing and fat mass was assessed by impedance. Women were examined twice at 6-month intervals, once while taking ORT and once after an oestrogen washout period. Two different forms of ORT were used oral and transdermal. IGT was found in 50% of TS subjects, this increased to 78% when ORT was administered. There was no

difference between modes of administration. Despite a marked increase in IGT incidence authors were unsure of an oestrogen effect due to the small sample size and disagreement with studies in the menopausal women in the general population; mentioning that variation is assessing measures or the known negative effect of progestin may have altered study results (Gravholt, Naeraa, et al., 1998). Fat-free mass was also higher within the TS group.

In 2006 Alves et al., studied 9 non-obese women with TS (mean age 23 ± 4.9) taking equine oral oestrogens (Alves, Gallichio, & Guimaraes, 2006). Body composition of the was assessed via DEXA and insulin measured using an IV glucose infusion. Women were then switched to a transdermal ORT and the measures repeated after one-year treatment. Alves (2006) found no significant difference found between insulin tolerance, however, transdermal ORT was found to be associated with favourable body composition changes such as lean body mass (Alves et al., 2006).

The largest of these studies was conducted by Torres-Santiago (2013). Torres-Santiago et al., found in a group of 40 girls with TS (mean age 16.7 ± 1.7 years) that if oestrogen was adequately replaced there was no significant difference in metabolism markers when comparing oral and transdermal ORT (Torres-Santiago et al., 2013). Although these reports are consistent in reporting defects in insulin secretion, small cohort sizes and differences in cohort mean age prevents conclusions regarding the effect of oestrogen being drawn.

Turner Syndrome-associated diabetes mellitus: evidence for genetic involvement

Many of the aforementioned studies have performed analyses of glucose homeostasis in TS; reported an increased association between the 45,X karyotype subgroup and adverse outcomes (Salgin et al., 2006).

In the 2008 study by Bakalov et al., the same women who undertook an OGTT and autoantibody tests were also submitted for genotyping and gene expression profiling by Quantitative Polymerase Chain Reaction (qPCR). Gene expression analysis indicated that those with 46,X,i(Xq) had increased Xq transcripts due to the triploidy of Xq. It was postulated by authors the q arm may contain potential DM-risk genes. Gene transcripts IGF2, GAD, and CRP were elevated within the

isochromosome group suggesting a pro-inflammatory state. A total of seventeen genes were overrepresented in those with 46,X,i(Xq) versus those with 45,X with known involvement in; beta-cell biology and survival, autoimmunity, insulin processing, and signalling. Over-expressed genes were not limited to the X chromosome. Several q arm genes were also found to be down-regulated such as *XIAP* (Xq25). *XIAP* is an inhibitor of apoptosis liver beta-cells from cell death (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008).

Bakalov at el., further reported that insulin deficiency was highest in those with 45,X and was increased more so in women with the isochromosome. Evidence for which, was supported by an increased prevalence of DM in those with Xp deletions (23%) versus those with 45,X (18%). Bakalov. suggested that genes located in PAR1 of the p arm that escapes X-inactivation could be involved in DM pathogenesis due to haploinsufficiency. The authors concluded that the DM phenotype observed in TS was more reflective of MODY, due to the lack of autoimmunity and reduced insulin secreted caused by two events; haploinsufficiency of genes involved in beta-cell function and glucose sensing alongside the over-expression of Xq genes that escape X-inactivation in the case of the isochromosome (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008).

Recent advances in technologies have allowed for a more in-depth examination of the X chromosome. In 2014, fibroblast models were used to compared the methylation patterns of 45,X against those with 46,XX. Methylation patterns were found to be different between the two groups of X-linked genes. Several autosomal genes were also found to have a differential methylation pattern including some linked to glucose sensitivity (Rajpathak & Deobagkar, 2014).

Trolle et al., (2016) also studied methylation and gene expression patterns in women with 45,X using RNA based technologies. The study found global hypomethylation of both *cis-* and *trans*-elements of the X chromosome. The authors concluded that widespread hypomethylation of promoters would likely impact gene expression regulation and therefore differential gene expression. Alterations to DM-associated gene methylation were also described but not disclosed (Trolle et al., 2016).

Turner Syndrome-associated Diabetes mellitus: evidence for lifestyle risk factors

Few studies have been conducted regarding lifestyle choices for individuals with TS. Gravholt et al., reported low levels of fitness calculated by maximal aerobic capacity (VO2max) (Gravholt, Naeraa, et al., 1998). A later study by the same research group investigated; glucose metabolism, adipokines, endothelial adhesion molecules along with body composition in forty-four women with TS (42.5 years ± 9.7). Of note there were body composition changes, such as low lean mass and increased adiposity detected by dual X-ray absorptiometry scans and were found to influence V02max, circulating hormones and insulin sensitivity (Gravholt et al., 2006). The authors concluded that a combination of increased visceral fat and changes to hormone profiles such as leptin would lead to an increased risk of IR. In regards to physical exercise tolerance, Tancredi et al., found that woman with TS physical cardiac changes which led to reduced maximal aerobic capacity and did not tolerate exercise as well as controls (Tancredi et al., 2011).

Obesity is a global health issue defined as a BMI ≥25kg/m². Hanew et al., observed obesity prevalence using BMI across 3 age groups in those with TS who had received GH treatment (n= 492) compared to the general female population. BMI was higher across the three TS age groups compared to the female general population. Between the three TS age groups, BMI did not consistently increase. The authors concluded obesity onset occurs early in those with TS and does not increase in prevalence with age (Hanew et al., 2016). However, short stature confounds the height-based BMI calculation making it difficult to interpret in TS. Obesity in TS has two major contributors' short stature and biology anomalies affecting metabolism (*see previous*). Therefore, until a height corrected BMI calculation exists it may be more representative of the TS-obesity phenotype to use other observations such as body composition. A small observational study found those with TS had a lower muscle mass along with increased whole-body fat and truncal fat percentage when compared to controls when corrected for height (Salgin et al., 2006). It is important to note that lowered muscle mass may affect overall metabolism. A 2017 study used indirect calorimetry to measure Resting Energy Expenditure (REE) in 20 TS subjects compared to matched controls. TS subjects were found to have an altered body

composition but also a significantly lowered REE (Sifuentes et al., 2017). It was unclear to the authors how the exact relationship between obesity and energy expenditure when other factors such as ORT compliance, eating behaviours, energy expenditure, and insulin sensitivity have not been considered together.

In 2018, Lebanthal et al., conducted an evaluation of metabolic syndrome in TS (Lebenthal et al., 2018). The longitudinal and cross-sectional data was derived from 98 women with TS. Researchers found that there was a significant increase in BMI through the life course of TS. Although ageing did play a large factor in comorbidity development, obesity was found exacerbated these issues such as hypertension and impaired glucose tolerance (Lebenthal et al., 2018).

Turner Syndrome-associated diabetes mellitus: evidence summary

In summary, research into TS-associated DM has produced conflicting results. The exact aetiology of Turner Syndrome-associated Diabetes mellitus remains unclear, largely due to the exclusion of Diabetes from studies and different study methodologies (table 1.4).

Genome-Wide Association studies relating to diabetes

When researching disease states such as T2DM using gene technologies there are a few approaches such as array-based studies such as Genome-Wide-Association Studies (GWAS). GWAS, one of cheapest technologies available, uses SNPs throughout the genome to identify variants directly or indirectly involved in disease pathogenesis. Studies can be hypothesis free or case-control based (Tam et al., 2019). GWAS has proven fruitful in identifying polymorphisms associated with the highly heritable T2DM but unfortunately the most significant variants were in non-coding gene regions leaving researchers uncertain of their pathogenic mechanism (Alonso, 2019; Tam et al., 2019).

The hypothesis stands that the haploinsufficiency of the X chromosome in TS results in the majority of comorbidities such as DM (Schoemaker et al., 2008; Stochholm et al., 2006; Sybert & McCauley, 2004). But, gene technologies in the general population have not consistently linked the X to DM. In 2000 Ehm et al., conducted a GWAS for DM in 4 American populations of different ethnic backgrounds. Located on the q arm of the X chromosome D12S853 was

identified in the American Caucasian population, with a logarithm odd score of 2.99 and was associated with diabetes and IGH (Ehm et al., 2000).

A more recent reanalysis of existing GWAS data from 70,127 T2DM cases, led to the identification of a rare variant located on Xp23, rs146662075 (Bonas-Guarch et al., 2018). The SNP was associated with a two-fold increased risk of T2DM in males.

Located within an enhancer, rs146662075, is associated with the expression of Angiotensin II receptor gene (*AGTR*) in T2DM (Bonas-Guarch et al., 2018). *AGTR* has been show to modulate insulin sensitivity and exhibits allelic specific activity in muscle cells in animal models (M. Liu, Jing, Wang, Liu, & Yin, 2015; Shao, Zucker, & Gao, 2013).

To prove the phenotypic effect of risk alleles identified through GWAS, knock-out mice are often implemented. Insulin receptor substrate 4 (*INSR4*) has been mapped to Xp22.3 - 23. Kadowaki (2000) observed a knock-out mouse model deficient in insulin receptors. The mice who were indistinguishable from their wild type or heterozygous littermates at birth, but went on to developed ketoacidosis and elevations in triglycerides and fatty acids which later led to hepatic steatosis. Pups also displayed insulin resistance (Kadowaki, 2000). Although *INSR4* would make for an interesting gene to model in TS, mouse models of TS are yet to be successful in displaying the full human phenotype (Probst, Cooper, Cheung, & Justice, 2008).

Despite some links to the X chromosome, there is a distinct lag in identifying casual variants to the X; only around 242 of all 734 GWAS conducted between 2005 - 2011 included the X chromosome (Konig, Loley, Erdmann, & Ziegler, 2014; Wise, Gyi, & Manolio, 2013). The main reasons for exclusion of the X include; a lack of array coverage, differences in gene/ variant number as well as Minor Allele Frequency (MAF) when compared to autosomes and a lack of power to detect change (Wise et al., 2013).

Commercial diabetes mellitus arrays

Commercial DM specific arrays are now available. Arrays consist of well-characterised polymorphisms which have been documented found to be in a higher frequency in those with T2DM compared to the general population. Arrays can be used on a general population basis or in individuals who are identified as having an increased risk of disease development (Palomaki, Melillo, Marrone, & Douglas, 2013). It is more common for commercial panels to be tailored towards T2DM but similar panels exist for T1DM, which has less pronounced inheritance pattern. Arrays have proven useful in distinguishing DM subtypes.

Combining both T1DM and T2DM associated SNP panels Mishra et al., (2017) sought to establish if LADA was genetically distinct from T1DM. Genetic signals from both T1DM and T2DM were found to be involved in LADA, however, LADA was found to be more similar to T1DM. Interestingly it was found that there were fewer risk alleles associated with childhood-onset of T1DM, which may account for the later age of presentation (Mishra et al., 2017).

Literature review discussion

The purpose of the review was to examine TS and associated conditions autoimmunity and DM. Women with TS have an increased risk of DM and reported IGT. Several reports have shown a marked reduction in first phase insulin response to glucose possibly induced by an intrinsic beta-cell defect which may be responsible for the increased prevalence of IGT (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008; V. K. Bakalov et al., 2004; Gravholt, Naeraa, et al., 1998; Hjerrild et al., 2011; Ibarra-Gasparini et al., 2018). Alongside this there well-established IR and decreased IS, believed to be intrinsic to TS due their presence in younger subjects with TS (Caprio et al., 1991; Salgin et al., 2006).All of which seems to be independent to obesity and oestrogen (Alves et al., 2006; Gravholt, Naeraa, et al., 1998; Torres-Santiago et al., 2013). But could be exacerbated by obesity and physical differences which may mean those with TS do not readily removed glucose from the bloodstream (Gravholt, Naeraa, et al., 1998; Hanew et al., 2016; Lebenthal et al., 2018; Salgin et al., 2006; Sifuentes et al., 2017; Tancredi et al., 2011). From a genetic perspective more evidence is needed to understand whether it is simply just the missing X chromosome or if is the combination of the missing X along with an individual acquiring secondary

genetic 'hits' that causes impaired beta-cell function (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008).

Autoimmunity is prevalent in TS with hypothyroidism and thyroid autoimmunity being common (Grossi et al., 2013; Mortensen et al., 2009). The review has discussed how TS associated DM many not fit into T1DM or T2DM distinctions exactly and shares features of both types and currently there is limited data available regarding the prevalence of DM-specific autoantibodies. Future work should focus on identified risk karyotype groups such as the isochromosome and monosomy X.

Advancements in gene technologies and the ever-falling costs could prove useful in the future research of TS associated DM. It is proposed that given the increased frequency of DM in those with TS gene technology may allow us to identify risk loci, distinguish TS-associated DM from other subtypes and identify novel pathogenic mechanisms. Given the global DM epidemic findings relating to diabetes in TS may prove useful to the wider population. The study of *TIMP1* serves as a great example of the potential of such research projects (Corbitt et al., 2018).

In conclusion in order to characterise the DM phenotype in TS an approach taking into consideration both genetic, biological and environmental factors is necessary to determine which factor most influences DM-risk in TS.

Chapter II

Research aims & hypothesis

Drawing on the deficit in knowledge surrounding TS-associated DM, the overall aim of these works was to characterise the factors influencing glucose homeostasis and TS-associated DM. All health outcomes such as TS-associated DM have two major components. The first is the influence of the missing X chromosome and the second is oestrogen deficiency. The primary objectives were to;

- Examine the relationship between TS-associated karyotypes and adult health outcomes. With a sub-aim of statistically clarifying the previously reported association between the isochromosome karyotype and DM-risk.
- Examine the relationship between the induction of puberty, GH therapy, and long-term oestrogen use and previously established health outcomes.

It was hypothesised that TS-associated DM is neither T1DM or T2DM as experienced by the general population and will have a unique profile. To characterise the factors influencing glucose homeostasis and TS-associated DM the second set of objects were set;

- Conduct oral glucose tolerance tests in those with previously identified DM-risk karyotypes; 45,X, 46,X,i(X), 45,X/46,X,i(X) and 45,X/46,X,r(X).
- Perform fasting blood tests in those with established DM attending UCLH.
- Collect all information pertaining to the DM-risk such as family history, anthropometrics and ethnicity.
- Assess beta-cell function through C-peptide measurements.
- Identify the prevalence of DM-associated autoantibodies and identify the role of autoimmunity in the pathogenesis of TS-associated DM.

The final set objects aimed to characterise the genetics of TS-associated DM.

- Conduct a pilot genomic study to establish if there is an over-representation of previously reported common T2DM variants in those with TS-associated DM.

Chapter III

Methodologies & statistics

Life course projects & ethical approval

The Turner Syndrome Life Course Project (TLCP) was established in 2014, to document the medical health outcomes for adult women with TS. A comprehensive questionnaire was developed in tandem to collect data on the psychosocial aspects of TS. Questionnaire topics ranged from; diet and exercise, education and job status and sexual function.

Patient involvement was a vital component in the development for the TLCP. All aspects of the TCLP were developed in conjunction with the Turner Syndrome Support Society (TSSS). Furthermore, a panel of women with TS was compiled to review questionnaire contents and format. The peer-review identified that women with TS sometimes struggle to interpret questions; most issues surround the diet and exercise portions of the questionnaire. The peer-review allowed us to address this issue in the early stages of development, leading to the final questionnaire being comprised of both standardised and TS-specific questions adapted from standardised questionnaires.

In 2015, the Reproductive Life Course Project (RLCP) was established to expand the same methodology of the TLCP to other rare endocrine disorders such as POI. The RLCP also encompassed DNA collection and analysis.

Ethics applications for both projects were developed and managed using the Integrated Research Application System (IRAS) by myself. I also created all supporting documentation such as the protocol, participant information sheet and consent form. Studies 1 and 2 were conducted under the TLCP whilst all other studies were conducted under the RLCP. UCLH sponsorship of the projects was sought and approved. Once sponsorship was established both projects were approved by the Chelsea Research Ethics Committee (TLCP reference: LO/2174; RLCP reference: 16/LO/0682). In the case of the RLCP several

amendments were made to include approval for a UK-wide pregnancy and fertility audit for women with TS and Congenital Adrenal Hyperplasia. Subsequent amendments were also approved by the Chelsea Research Ethics Committee. The Health Research Authority (HRA) came into effect sometime during the project, approval was of which was secured in both instances. HRA was also updated with each subsequent project amendment. *Appendices 1-3 contains the most recent and relevant documentation relating to the above*.

University College London Hospitals adult Turner Syndrome clinic

UCLH has had a dedicated adult TS service for over 20 years. The multidisciplinary clinic, consisting of both endocrinology and gynaecology, undertakes annual health surveillance and management of women with TS. Since the initiation of the clinic over 835 women have attended accumulating over 8,000 clinic visits. This is believed to be the largest TS data set in the world.

Recruitment & subjects

Recruitment took place between 2014-2019. Figure 3.1 summarises the recruitment process. All women approached to take part in the Life Course Projects attended UCLH Disorders of Reproductive Development (DRD) clinics. An introductory letter and patient information sheet were posted in advance to clinic appointments. Later, when the women attended clinic I approached them, reintroducing the study and answering questions regarding their participation. Most women were recruited and consented under the TCLP, the older of the two life course projects. When the DNA extraction and analysis portion of the RLCP were approved women, who were applicable, were either consented or reconsented under the RLCP. All women with POI were recruited under the RLCP. Questionnaires were either posted or given to participants after the consent process.

Recruitment & subjects: controls

When studying biological processes or disease states in TS it is important to eliminate oestrogen deficiency as an influencing factor. Individuals with POI serve as robust controls group in this instance as like women with TS, they can experience primary amenorrhoea and therefore oestrogen deficiency. Women with POI were recruited for study 3 for this purpose.

Figure 3.1 flowchart of the Turner Syndrome and Reproductive Life Course Project recruitment process.


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┌─────────────────────────────────────┐
│      Life Course Projects           │
│      Consent Process                │
└─────────────────────────────────────┘
                 │
                 ▼
┌─────────────────────────────────────────────────────────┐
│                  Consent process                        │
│  • Invitations and participant information sheet sent   │
│    in advance to clinical appointment at UCLH.          │
│  • Potential participants were approached in clinic     │
│    for participation.                                   │
└─────────────────────────────────────────────────────────┘
                 │
                 ▼
┌─────────────────────────────────────┐
│      Turner Syndrome Life           │
│      Course Project (2014)          │
│      • Medical outcomes             │
│      • Questionnaire                │
│      • Women with TS only           │
└─────────────────────────────────────┘
                 │
                 ▼
┌─────────────────────────────────────────────────────────┐
│           Reproductive Life Syndrome                    │
│           Life Course Project (2015)                    │
│  • Medical outcomes                                     │
│  • Questionnaire                                        │
│  • Applicable TS women were reconsented under the       │
│    RLCP for blood sample collection and analysis        │
│  • Women with TS and POI                                │
└─────────────────────────────────────────────────────────┘
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Inclusion criteria

The inclusion criteria for all methodologies was a confirmed diagnosis of TS or POI with a current age of above 16 years old currently attending UCLH clinics.

Exclusion criteria

Excluded were those women did not wish to participate and those with late-onset secondary amenorrhea in the case of POI or those women who no longer attended UCLH.

Life course project data & Turner Syndrome adult health outcomes

Parameters for analysis were chosen to represent common morbidities of TS based on previously published literature (Al Alwan, 2014; Gravholt, 2004; Grossi et al., 2013; Mortensen, Andersen, & Gravholt, 2012; Schoemaker et al., 2008; Stochholm et al., 2006; Sybert & McCauley, 2004; Yesilkaya et al., 2015). At each clinic visit data for each health outcome was recorded. Clinical data were grouped to represent common pathogenesis associated with TS: autoimmunity was represented by use of hypothyroidism treatments, positive thyroid autoantibodies (TPO) and coeliac disease; metabolism was represented by liver dysfunction alkaline phosphatase (ALkP), alanine transaminase (ALT) and gamma-glutamyl transpeptidase (GGT); diabetes was represented by use of diabetes treatment and HbA1c; lipid dysfunction was represented by non-fasting lipid profile total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL) and triglycerides; bone outcomes was represented by bone density measurements by DEXA and the use of osteoporosis treatment but excluded the use of vitamin D; deafness by use of a hearing aid; cardiovascular outcomes by blood pressure measurements, the use of antihypertensive treatments, presence of a bicuspid and aortic size index (ASI). ASI was calculated using; a*ortic size/body surface area* (BSA). The Mosteller equation was used to calculate BSA; $\sqrt{(height(cm)\, x\, weight(kg)/3600}$.

Drug treatments were recorded for the most common comorbidities such as hypothyroidism, diabetes mellitus, hypertension, depression and osteoporosis, excluding vitamin D use. Age of first receiving treatment was taken as a marker of the age of disease onset. Although it should be stated that age of treatment initiation is an estimation of disease onset as the disease may have been left untreated for some time. For instance, in the case of DM, if HbA1c is elevated at just one clinical visit a patient may be advised to try a healthy living approach before the use of anti-diabetics.

In study 3, additional anthropometric data were obtained including body fat using impedance (Tanita Body Composition Analyzer BC-418 MA) and waist circumference (cm). Family history of DM and ethnicity was also recorded. Furthermore, insulin resistance was calculated using the following HOMA-IR formula; fasting insulin (microU/L) x fasting glucose (nmol/L)/22.5;.

Cross-sectional data

Cross-sectional data was taken from the latest clinic data available for each of the aforementioned biochemical health outcomes such as BMI and liver enzymes. This extended to the presence or absence of comorbidities as the time of the analysis such as hypothyroidism and DM. Also recorded were the commencement of paediatric treatments such as GH therapy.

Longitudinal data

Data was available from 1994 to 2018. Data were collected retrospectively from when the patient was aged 16 or from their first clinic visit to UCLH. When data was not available in records, patient recall was implemented. It was not an objective to test treatment effectiveness therefore, clinic visits were an individual was undertaking treatment for a specific co-morbidity was excluded from the final analyses. For instance, blood pressure data were not analysed from visits that took place after a diagnosis of hypertension. The same was also true for DM, which related mostly to study 1 and 2.

Study specific methodologies

Various methods were implemented in a multifaceted approach to characterising TS-associated DM. Each analysis was conducted at a different time point therefore, sample and data size set varied throughout. The following describes each methodology. Methodologies relating to the pilot genetics study can be found in chapter VI.

Study 1 methodology: The relationship between Turner Syndrome-associated karyotype & health outcomes

The following was published 2017 (Cameron-Pimblett, La Rosa, King, Davies, & Conway, 2017) and therefore is summarised (*see appendix for full text).*

The clinics at UCLH have records of 782 women with TS of whom 20 (2.5%) declined consent. For 762/782 individuals, the inclusion criteria for this report were a confirmed diagnosis of TS and a recorded TS karyotype. The original karyotype was not always available and therefore repeated or retrieved from local cytogenetic centres; St Thomas and Guys, Northwick Park, and Great Ormond Street hospitals. Karyotypes were available in 656 (78.1%) individuals.

Previous reports have indicated that those with 45,X experience the most adverse outcomes when compared to all other groups especially those with 45,X/46,XX as a consequence of global haploinsufficiency of the missing X chromosome (Al Alwan, 2014; Gravholt, 2004; Grossi et al., 2013; Mortensen et al., 2012; Schoemaker et al., 2008; Stochholm et al., 2006; Sybert & McCauley, 2004; Yesilkaya et al., 2015). In study 1, the 45,X group outcomes were used as a reference to compare all other karyotype groups against.

Study 2 methodology: The relationship between first oestrogen exposure, long-term oestrogen use, growth hormone & health outcomes

The following is published (Cameron-Pimblett et al., 2019) and therefore is summarised (*see appendix for full text*). The aim of study 2 was to examine the influence of paediatric treatment such as timing of oestrogen exposure and GH, and long-term oestrogen use on previously established health parameters (Cameron-Pimblett et al., 2017).

Investigation into the timing of first oestrogen exposure

Primary amenorrhoea occurs in around 95% of those with TS (Pasquino et al., 1997). Age at which oestrogen was first prescribed was taken as the age of first oestrogen exposure, which was determined by review of paediatric notes and patient recall when previous notes were not available.

Assessment of the impact of growth hormone therapy

GH is commonly prescribed in TS to improve final adult height and is taken during childhood years (Gravholt et al., 2017). In this assessment in the effect of GH on adult health outcomes 327 who did not receive GH were compared to 426 who did. GH treatment duration affected adult health outcomes was also examined. Treatment duration data were available for 356/426 (83.6%).

Assessment long-term oestrogen replacement therapy

ORT varies over the lifespan for an individual with TS. For each clinic visit, the type of ORT was recorded. The final data set consisted of 6,679 clinic visits from 599 individuals with both primary and secondary amenorrhoea.

Three ORT subgroups were used by women in the series: Combined Oral Contraceptive pill (OCP), Oral Oestrogens (estradiol valerate & conjugated equine) (OE) and Transdermal estradiol (TE). A small sub-group of women used Ethinyl-estradiol these women were included in the OCP group.

Study 3 methodology: characterisation of glucose homeostasis & diabetes mellitus in adults with Turner Syndrome

To assess glucose homeostasis in risk karyotype groups, a standard OGTT protocol was implemented (*see appendix for protocol*). Previous studies suggest there is an excess of DM in those with isochromosome X (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008). In addition, the results of study 1 suggested a statistical association between the ring chromosome and DM. To clarify these previous associations, recruited were women with either an isochromosome or a ring chromosome karyotype. Furthermore, women with 45,X were also recruited to compare the incidence of IGT and DM.

Fifty-five women were identified as having either T1DM or T2DM on the UCLH TS database. Of which, 29/55 (52.7%) subjects were recruited. Twenty-seven women were submitted for a fasting glucose, insulin and lipid; 2/29 subjects were unable to give for blood sample but provided samples DM-associated autoantibody testing. The remaining 26/55 (47%) were lost to follow-up and/or could not be contacted. In both groups, consenting subjects, blood tests were conducted after 10 hours of fasting and in the case of established DM subjects before the administration of anti-diabetic medications.

Furthermore, 16 women were recruited with POI. It has long been established that women with TS have insulin deficiency and abnormal glucose homeostasis when compared to women with POI controls (V. K. Bakalov et al., 2004). Women were selected based on their; age and if they had primary amenorrhea to establish an oestrogen deficient reference range for biochemical parameters.

Assessment of beta-cell function through c-peptide measurements

It has been hypothesised that women with TS have reduced beta-cell function, thus making them more at risk to developing DM (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008). To assess c-peptide, serum was extracted, by centrifugation, to quantify C-peptide; the prohormone to insulin. Extracted blood serum was sent to the Great Ormond Street Hospital. The standard C-peptide reference range was 260-650 pmol/L.

Identifying the prevalence and influence of diabetes mellitus-associated autoantibodies in women with Turner Syndrome

To evaluate the role of autoimmunity in IGT and DM pathogenesis blood samples were obtained from subjects outlined previously. Extracted serum was sent to Exeter Clinical Laboratory International for DM-associated autoantibody testing; GAD, IA-2, Zinc Transporter 8 (ZnT8) using a standardised protocol. Positive titre test for autoantibodies were; GAD+ ≥11 U/mL, IA-2+ ≥ 7.5U/mL and ZnT8+ if subject age is ≤30 years 65 U/mL or if the subject's age was ≥31 years ZnT8+ ≥10 U/mL.

Statistics: general

Various analyses were implemented for each methodology using statistical software SPSS version 22 (SPSS Inc., Chicago, IL, USA). The following is a general description of the analysis applied to studies 1-3. Statistics relating to pilot genetics study can be found in chapter VII.

Confounding variables

Over-time medical practices have changed and due to the retrospective nature for many of the analyses conducted individual current age confounded the data. This mostly related to studies 1 and 2. This was the case for BMI, as a reflection of those individuals who did not receive GH during paediatric years. Therefore, an individual's current age and BMI were controlled throughout. In the analyses relating to bone outcomes a further control for height was applied, again to control for those who did and did not received GH therapy.

Data normality and missing data

Data were tested for normality using the Shapiro-Wilk test. Diastolic and systolic blood pressure, liver enzymes, HbA1c, LDL and triglycerides did not show a normal distribution and were log-transformed for analysis. Geometric means of the data along with confidence intervals were used as results. Data was then anti-logged from the geometric mean for presentation and results.

Some data points were missing as health surveillance has evolved. Less than 10% of data were missing for most variables. Other variables were missing up to 20% and included bone mineral density t-score, ASI, hearing aid use, serum GGT, coeliac and thyroid antibody results. Reflecting changes in clinical practice and the recent inclusion of GGT into annual health surveillance.

Testing for group differences

Two basic statistical analyses were implemented to test for group differences. For continuous variables such as weight, an ANOVA was used (studies 1-3). Furthermore, if three or more groups were present a Tukey post hoc was used to identify group differences. For categorical variables such as antibody status a chi-squared was used (studies 1-3).

In the case of study 1, the chi squared analysis was used in a slightly different fashion for continuous variables. Continuous variables were converted into a binary format, allocating those in the upper quartile to represent those within the cohort with more adverse outcomes compared to the lowest three quartiles combined. In the case of bone mineral density t-scores, the lower quartile was taken as an adverse outcome. Table 3.1 shows each health parameter against the UCLH reference range and the calculated 'at-risk' cut off points. Each karyotype subgroup was then compared to 45,X which was used as a reference for all TS related health outcomes.

Study 3 implemented both ANOVA and chi-squared analysis at various points. Results from those with IGT and DM, both newly diagnosed and established were combined in order to improve statistical power when testing for group differences. If group differences were detected a subsequent within TS analysis was conducted to distinguish the IGT from that of the DM group.

A small number of women were recruited with POI (n= 15) were recruited as controls. Such a small group size led to a data skew of DM-associated biochemical parameters such as insulin. Instead of an ANOVA, a Krustal-Wallis test was used to compare group medians and interquartile ranges (IQR) calculated. Each group was compared separately.

Table 3.1 shows each of the Turner Syndrome-associated health parameters with the local reference range and calculated 'at risk' group cut-offs. The quartile cut point for those in the top quartile or bottom quartile in the case of bone t-score indicates the "at-risk" range. In most instances, the quartile cut point approximates to the upper boarder of the reference range.

Health Outcome	Local Reference Range	Quartile cut point for 'at- risk' group
BMI	≤25	≥29
Diastolic Blood Pressure	≤85 mmHg	≥83 mmHg
Systolic Blood Pressure	≤130 mmHg	≥135 mmHg
HbAc1	4.0-6.0 %	≥5.6 %
ALT	10-35 IU/L	≥39 IU/L
ALKP	35-104 IU/L	≥105 IU/L
GGT	6-42 IU/L	≥85 IU/L
Aortic Size Index	≤2.0 cm/m²	≥1.96 cm/m²
Hip T- Score	≤-2.5	≥-1.5
Spine T- Score	≤-2.5	≥-1.8

Assessment variable relationships

Correlations can be used to determine whether there is a relationship, negative or positive, between two continuous variables. Partial correlations were implemented in the assessment of BMD in study 2.

Study 3 specific statistics

Area Under the Curve

Area Under the Curve (AUC) is a calculation used in glucose homeostasis studies. AUC can be used as an index of whole glucose exertion (Sakaguchi et al., 2016). AUC was calculated using the trapezoidal method using plasma glucose concentration at each OGTT time point (Sakaguchi et al., 2016). As the AUC was calculated after OGTT outcome was known, means were generated for each group at each time point to account for missing data.

Receiver operator curve

In the general population; BMI, waist circumference, HbAc1 and fasting glucose are all used as markers for diagnosing DM or estimating DM-risk. However, Ibarra-Gasparini et al., highlighted a discordance between baseline glucose, glucose after 120 minutes and HbAc1 (Ibarra-Gasparini et al., 2018). Thus, suggesting that the sensitivity of such measures in detecting diabetes in TS may be reduced. With this in mind, constraints within the NHS means that the resources to conduct OGTT may not always be available and so clinicians need a way to triage at-risk patients from the TS population for an OGTT.

The results of women submitted for an OGTT were used in a Receiver Operator Characteristic (ROC) analysis of; BMI, waist circumference, fasting glucose and HbAc1. Data from those with established DM was excluded as women were currently undertaking treatment or lifestyle modifications. The ROC analysis uses sensitivity against specificity (1-sensitivity) to generate an AUC. The AUC can then be used to assess whether or not the assay is a good predictor of the outcome, which in the case of study 3 was IGT/DM. Models generating an AUC closer to 1 would indicate better models of outcome prediction. The plotted assay data is used to identify the data point closest to a sensitivity of 1. Once identified the coordinates can be used against the SPSS generate table to find cut-offs.

Linear regression analysis

A linear regression analysis was performed to identify which factors were involved in T2DM in TS. Those variables investigated were those found to influence T2DM in the general population; age, BMI and HOMA-IR.

Chapter IV:

Results of study 1: relationship between Turner Syndrome-associated karyotype & health outcomes

The aim of study 1 was to determine if there was an association between TS karyotypes and health outcomes when compared against the reference karyotype 45,X. The following was published (Cameron-Pimblett et al., 2017) and has been abbreviated here with tables and figures (see *appendix for full report*).

Seven karyotype groups were identified including five major; 45,X (41.6%); 45,X/46,XX mosaicism (15.7%); isochromosome X including mosaicism- 45,X,i(Xq) and 45,X/46,X,i(Xq) (18.8%); 45,X/46,XY mosaicism (10.7%) and ring X mosaicism- 45,X/46,X,r(X) (7.3%). Two minor subgroups were also identified including partial p or q X deletions (3%) and complex karyotypes involving translocations or complex mosaicism such as 45,X/46,XX/47,XXX (3.8%, table 4.1). Due to small sample size and group heterogeneity 45 women (6.8%) with partial Xp deletions, Xq deletions and complex forms of mosaicism with multiple cell lines were excluded. The final cohort size was 611. The study retrospectively accumulated 8,065 clinic visits representing 25,582 patient-years of follow-up.

Table 4.1 shows the recorded karyotype distribution for the University College London Hospitals adult Turner Syndrome cohort.

Karyotype	Sample Size
Monosomy 45,X	270 (41.6%)
Mosaic 45,X/46,XX	103 (15.7%)
Isochromosome X (all) 45,X,i(X) 45,X/46,X,i(X) mosaic	120 (18.8%) 28 (4.3%) 92 (14%)
Mosaic 45,X/46,XY	70 (10.7%)
Ring X: 45,X/ 46,X,r(X) mosaic	48 (7.3%)
Subtotal	611
Complex: 45,X/47,XXX mosaic X autosome translocations 3 or more cell lines i.e. 45,X/46,XX/47,XXX	20 (3.0%)
Partial X Deletions: 46,X,der(X) 46,X,del,X(q) 46,X,del(p) 45,X/46,X,del(X)	25 (3.8%)
Total	656

Paediatric outcomes for karyotype subgroups

Mean age of the cohort was 32.9 years (range 18.1 - 70.3 years) and median age of diagnosis was 10.1 years (range birth-61 years). Median final height was 1.49m (range 1.26m - 1.77m). The mean age of diagnosis was 10.1 years old. The prevalence of secondary amenorrhoea was 14.4%, whilst the prevalence of spontaneous menstrual cycles at the clinic last visit was 11%. Table 4.2 summarises the paediatric cohort outcomes for each karyotype subgroup. The earliest mean age of diagnosis was found within the 45,X group, 8 years. As expected, the latest age of diagnosis was found within the 45,X/46,XX group, age 13.2, (p= ≤0.01). Believed to reflect the pathway to diagnosis due to secondary amenorrhea and delayed puberty as opposed to severe height deficiency or congenital anomalies.

Table 4.2 shows the paediatric outcomes for 611 women from the University College London adult Turner Syndrome cohort. ANOVA analysis was used to compared each subgroup to 45,X separately. Displayed are the standard deviations. * p= ≤ 0.05 and **p= 0≤0.01.

	45,X (n= 270)	Mosaic 45,X/46,XX (n= 103)	Isochromosome Xq (n= 120)	Mosaic 45,X/46,XY (n= 70)	Ring X (n= 48)
Age of Diagnosis	8 ± 7.2	13.2 ± 9.2**	11.1 ± 7.8**	10.8 ± 10.5*	12. 9± 6.8**
Height (m)	1.49 ± 0.07	1.51 ± 0.07**	1.49 0.07	1.5 2 ± 0.08**	1.46 ± 0.06**
Primary amenorrhoea (%)	88.5	41.7**	78.3**	81.4	58.3**

Adult Health parameter outcomes for karyotype subgroups

Each clinical outcome pertaining to each karyotype subgroup compared to 45,X is presented in table 4.3.

45,X/46,XX

45,X/46,XX mosaic had the lowest frequency of comorbidities compared to 45,X. The result was expected and characterised by a significantly lesser incidence of primary amenorrhoea, obesity and hypertension when compared to 45,X (p= ≤0.01).

Isochromosome (Xq)

The isochromosome (Xq) karyotype group was found to have similar outcomes to 45,X. Except in the case of bicuspid valve; were there was a reduced incidence of bicuspid valve (11.7% vs 22.2%; p= ≤0.01) and upper quartile ASI (10.7% vs 23%; p= ≤0.01). Unlike other cohorts, no excess of severe hearing loss, congenital heart disease, diabetes mellitus or autoimmunity was found when compared to 45,X. In addition, a trend of elevated HbAc1, but did not achieve significance.

To examine the effect of mosaicism in the isochromosome (Xq) group, 28 cases of non-mosaic 46,X,i(Xq) were compared to 45,X. The non-mosaic 46,X,i(Xq) group was found to have significantly lower incidence of severe hearing loss (12.3% vs 17.5%; p= ≤0.01); bicuspid valve (10.9% vs 16.1%; p= ≤0.01) and thyroid antibodies (27.3% vs 31.1%; p= ≤0.01). The incidence of ASI (18.8% vs 23.0%; p= 0.85) and DM (5.6% vs 5.7%; p= 0.59), non-mosaic 46,X,i(Xq) did not significantly differ to 45,X.

45,X/46,XY

Height deficit was lowest in the XY group (1.52m ± 0.08 vs 1.49m ± 0.07; p= ≤0.01, table 4.3). The XY found had the lowest incidence of severe hearing loss (5.7% vs 23.3%; p= ≤0.01), hypothyroidism (14.3% vs 35.9%; p= ≤0.01) and reduced ASI (8.6% vs 23%; p= ≤0.01) compared to 45,X.

Ring chromosome X (r,X)

The ring chromosome group was the only group to display a severer phenotype when compared to the reference 45,X group. The data supported a previous finding of a greater height deficit (1.46m ± 0.06 vs 1.49m ± 0.07; p= ≤0.05) (Migeon, Luo, Jani, et al., 1994; Migeon, Luo, Stasiowski, et al., 1994; Van Dyke et al., 1992). Novel findings included an increased incidence of metabolic syndrome characterised by elevated HbA1c (25% vs 14.1%; p= 0.05) and ALT (33.3% vs 20%; p= ≤0.05). A similar, non-significant, trend indicating a metabolic defect in the ring chromosome group was seen in increased incidence of diabetes mellitus, elevated gamma GT and blood pressure (p= 0.16 & p= 0.15, respectively). In the case of bicuspid valve (4.2% vs 22.2%; p= 0.05) and severe hearing loss (10.4% vs 23.3%; p= 0.05) there was notably reduced incidence when compared to 45,X.

Table 4.3 summary of Turner Syndrome-associated comorbidities by karyotype subgroup (total n= 611). Participants' most recent visit data were used to take a cross-section. For continuous variables, the upper quartile was allocated to represent the "at-risk" group excluding bone mineral density T-scores were the lower quartile was used. A χ2 analysis was used to compare the prevalence of subjects in the "at-risk" group in the 45,X to each of the four karyotype subgroups using a χ2 analysis. *Represents p= ≤0.05 or **p= ≤0.01.

Health Parameter	45,X N= 270	Mosaic 45,X/46,XX (n= 103)	Isochromosome Xq (n= 120)	Mosaic 45,X/46,XY (n= 70)	Ring X (n= 48)
Current Age	34.6±12.1	29.8± 11.6**	33.0± 11.5	30.6±10.5**	33± 10.8
Audiology					
Hearing Aid Use (%)	23.3	6.8**	21.7	5.7**	10.4
Cardiovascular					
Hypertension (%)	20.7	7.8**	20.0	10.0	18.8
Diastolic blood pressure (%)	27.4	12.6**	25.8	18.6	35.4
Systolic blood pressure (%)	23.7	12.6*	30.8	18.6	29.2
Bicuspid valve (%)	22.2	9.7	11.7**	15.7	4.2*
Aortic size index (%)	23	10.7	13.3**	8.6**	18.8
Metabolism					
BMI (%)	11.5	1.9**	9.20	7.10	8.30
Diabetes mellitus (%)	7.0	1.9	6.7	4.3	12.5
HbAc1 (%)	14.1	8.7	22.5	14.3	25.0*
ALT (%)	20.0	16.5	28.3	22.9	33.3*
ALKP (%)	24.8	15.5	23.3	22.9	27.1
GGT (%)	21.9	9.7*	22.5	17.1	29.2
Mental Health					
Depression (%)	9.6	3.9	11.7	10.0	14.6
Autoimmunity					
Hypothyroidism (%)	35.9	26.2	31.7	14.3**	27.1
Thyroid antibodies (%)	15.6	3.9	11.7	5.7	6.3
Coeliac antibodies (%)	6.3	3.9	7.5	2.9	2.1
Bone					
Osteoporosis (%)	4.1	5.8	4.2	2.9	4.2
Spine T-Score <1.0 (%)	16.3	13.6	18.3	11.4	16.7
Hip T- Score <1.0 (%)	21.9	13.6	15.0	8.6	25.0

Chapter V:

Results of study 2: The relationship between first oestrogen exposure, growth hormone therapy, long-term oestrogen use & health outcomes

The aim of study 2 was to determine if paediatric treatments such as GH therapy and age of first oestrogen exposure influence health outcomes later in life. Long-term oestrogen exposure and differing modalities was also examined. The following report was published (Cameron-Pimblett et al., 2019).and the following is an abbreviated version *(see appendix for full report).*

Investigation into the timing of first oestrogen exposure

For those with primary amenorrhoea median (5th and 95th centiles) age of starting oestrogen was 14 years (5 - 23.4 years). Individual current age positively correlated with the age of first oestrogen exposure ($r = -0.40$, $p = \leq 0.001$). The correlation between current age and age of first oestrogen exposure indicated that over the life course of the clinic the mean age of oestrogen exposure has reduced.

BMD t-score for hip and spine had a negative association with oestrogen start age ($r = -0.20$ & $r = -0.22$ respectively, $p = \leq 0.001$). No other associations between adult outcomes and oestrogen start age. Figure 5.1 shows the age adjusted spine t-score values and age of first oestrogen exposure. Women who received oestrogen after the age of 20 were excluded. The figure demonstrates that those whom were first exposed to oestrogen at an earlier age were found to have higher t-scores later in life.

Figure 5.1 The plot shows adult bone density measurements of the spine (t-score) in women with Turner Syndrome presenting with primary amenorrhea against the age of first exposure to oestrogen for induction of puberty. Data points are adjusted for age, height and BMI. Best fit line and 95% confidence intervals for 786 BMD results in women with TS; ($r^2 = 0.04$, p= <0.05).
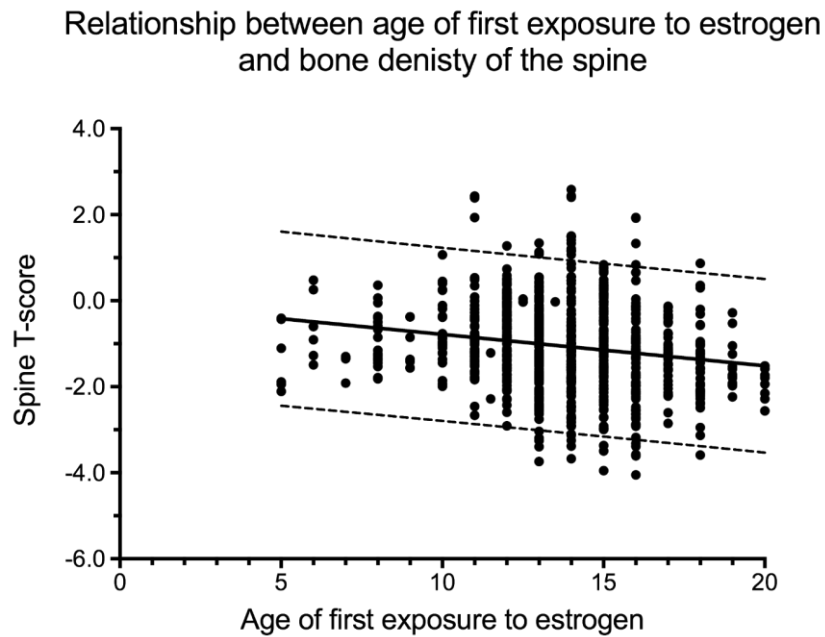
Figure 5.2 A plot of age and the percentage each of the 3 common types of Oestrogen Replacement Therapy; Combined Oral Contraceptive (OCP), Oral Oestrogens (OE), Transdermal Estradiol (TE) and those whom receive no treatment "none" averaged over in three-year age bands. The line indicated that by 57 years of age 50% of women have withdrawn from OE.
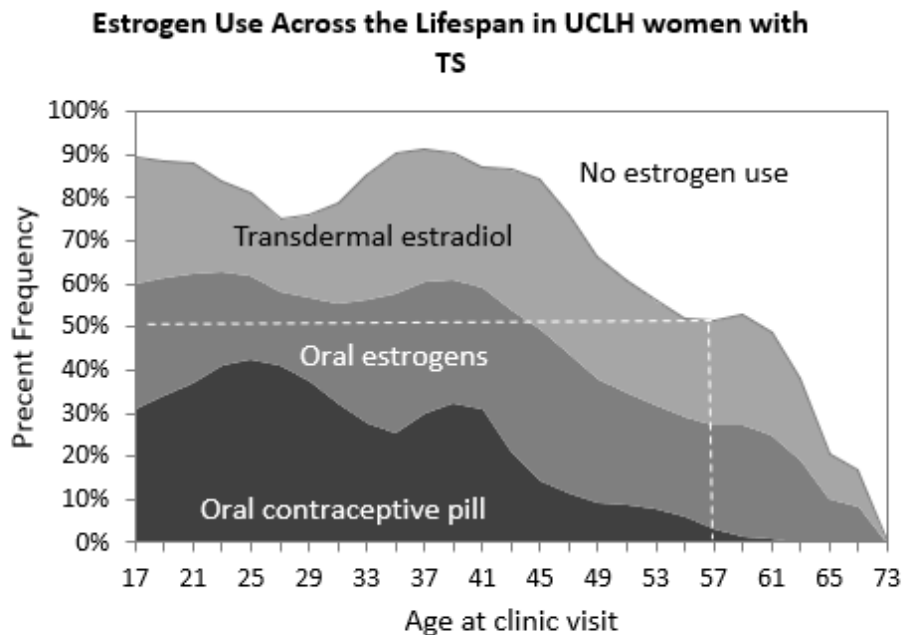
The impact of growth hormone therapy

Data regarding GH use in paediatric years was available in 753 women. A total of 56.6% of the cohort received GH therapy. For those whom received GH the median (range) age of starting GH was 5 (range 1 - 19 years). GH therapy had been received in childhood by 72% of those women under 35 compared to just 11% of women over 40. No associations were found between the use of GH or duration of treatment and TS adult health outcomes.

Assessment of long-term oestrogen replacement therapy

The median age (range) at each clinic visit was 31 years (16 - 73 years). The number of visits (n= 6679) relating to each ORT subgroup was OCP (n= 2226), OE (n= 3675) and TE (n= 778).

Figure 5.2 shows the relationship between ORT prescribing habits changes and age at visit. As expected, OCP use was common prescribed to younger women. After the age of 30 a change towards OE use is noted. ORT is withdrawn from the late 40's mimicking menopause in experienced by the general female population. Those few whom use oestrogen later in life are likely to be women who were making up of lack of ORT use earlier in life. Generally, there has been has been a decline in the OCP as a form of ORT in TS as prescribers have become of the risks associated with OCP use such as hypertension.

Table 5.1 shows the association between oestrogen subtypes and health outcomes. Similarly, BMI was found to be a greater in those using the TE verses those using the OE and OCP. Combined the data is suggestive of a prescriber bias as younger OCP or OE users have a lower BMI contrasting the typical older TE users whom have a higher BMI

Elevated liver enzymes; ALT, ALkP and GGT were found in those receiving TE when compared to other user groups (p= ≤0.001). Also noted within the TE user group was an elevation of HbAc1 (p= ≤0.01). Finally, blood pressure was higher in OCP user group compared to the OE and TE user groups (p= ≤0.001). Figure 5.3 shows blood pressure differences across the three ORT subgroups.

Table 5.1 summary of associations between oestrogen sub-groups; Combined Oral Contraceptive (OCP), Oral oestrogens (OE) and Transdermal Estradiol (TE) and adult TS health outcomes for those with both primary and secondary amenorrhea controlling for current age and BMI. Results are shown as mean and confidence intervals. *= significance at ≤0.01, **= significance at ≤0.001.

Health Outcomes (UCLH reference ranges)	Combined Oral Contraceptive (OCP)	Oral Estrogens (OE)	Transdermal Estrogen (TE)
Age	23.4 (23.1- 23.7)	33.2 (32.9- 33.5)	34.2** (33.3-35.1)
BMI	25.3 (25.1 - 25.5)	27.1 (26.9 - 27.3)	28.0** (27.5 - 28.5)
Alkaline Phosphatase (35- 104 IU/L)	82.2 79.6 - 84.7)	83.9 (82.4 - 85.5)	97.9**(93.8 - 102.1)
Gamma-Glutamyl Transpeptidase (6- 42 IU/L)	43.5 (39.1 - 48.3)	42.0 (39.5 - 44.5)	57.1** (49.9 - 65.3)
Alanine Transaminase (10- 35 IU/L)	27.1 (27.9 - 28.4)	28.5 (27.7 - 29.2)	34.8** (32.7 - 37.1)
HbAc1 (4- 6%)	7.3 (6.7 - 8.0)	6.9 (6.6 - 7.1)	7.7* (7.2 - 8.2)
Cholesterol (2.5- 5.0 mmol/L)	5.4** (5.3 - 5.5)	5.1 (5.0 - 5.1)	5.2 (5.0 - 5.3)
LDL (0- 3.5 mmol/L)	2.9** (2.8 - 3.0)	2.5 (2.5 - 2.6)	2.6 (2.5 - 2.7)
HDL (1.2-1.7 mmol/L)	1.8 (1.7 - 1.8)	1.8 (1.8 - 1.8)	1.8 (1.8 - 1.9)
Triglycerides (0.4- 2.3 mmol/L)	1.3** (1.2 - 1.3)	1.2 (1.1 - 1.2)	1.1 (1.0 - 1.2)
Aortic Size Index (≤2.0cm/ m²)	1.8 (1. 7- 1.8)	1.8 (1.8 - 1.9)	1.8 (1.7 - 1.8)
Spine T-Score (≥ -2.5)	-1.2 (-1.4 - -1.1)	-1.1 (-1.2 - -1.0)	-1.0 (-1.2 - -0.7)
Hip T-Score (≥-2.5)	-1.1 (-1.4 - -0.8)	-0.9 (-1.2 - -0.7)	-0.7 (-1.2 - -0.3)
Diastolic BP (≤85 mmHg)	75.5** (74.1 - 78.6)	73.1 (72.4 - 74.1)	74.3 (73.5 - 75.2)
Systolic BP (≤130 mmHg)	125.3** (124.5 - 126.2)	121.1 (120.5 - 121.6)	121.9 (120.8 - 123.3)

Figure 5.3 plot of diastolic and systolic blood pressures or each Oestrogen Replacement Therapy (ORT) user groups; Combined Oral Contraceptive (OCP), Oral ORT (OE), Transdermal (TE) mean and 95% confidence intervals for blood pressure measurements at each clinic visit in women with TS plotted against the type of oestrogen taken at the time. * indicates p= <0.05.

Blood pressure variation according to estrogen treatment group
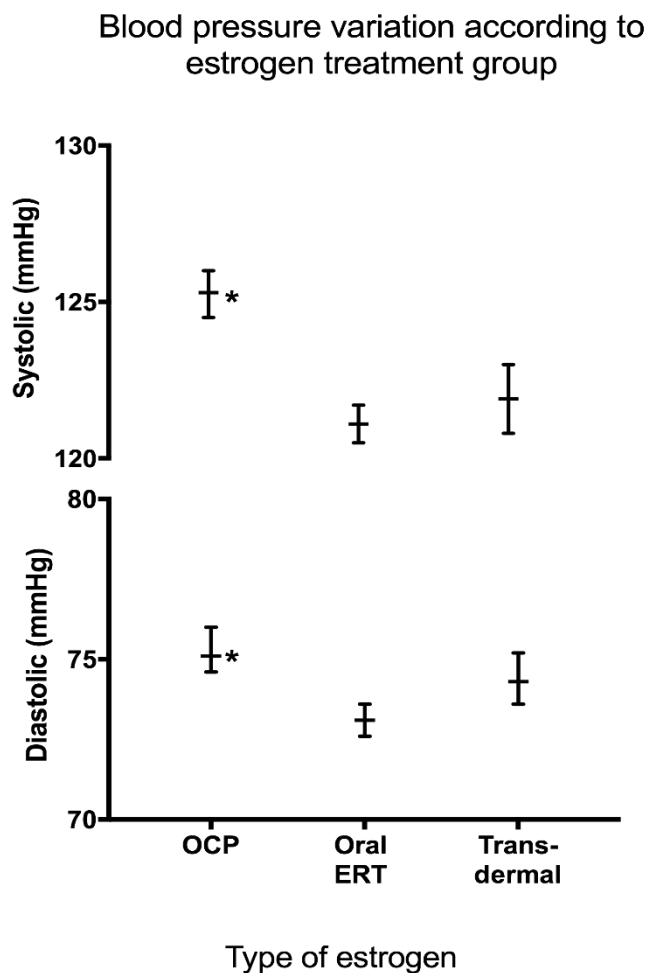
Chapter VI

Results of study 3: characterisation of glucose homeostasis and diabetes mellitus in adults with Turner Syndrome

This component of research aimed to undertake a more detailed characterisation of diabetes on women. The focus evolved because diabetes is one of the more important modifiable health risks for women with TS and because the pathogenesis is unclear with the possibility that greater knowledge might lead to new insights for diabetes in the general population.

Observations from study 1 in this thesis suggested that some karyotype subgroups may be at a greater risk of diabetes than others, but this was based on the diagnosis of pre-existing diabetes. The strategy in this part of research was to undertake a screen for glucose tolerance in risk groups with ring or isochromosomes compared to those with 45,X. Because of their heterogeneity 45,X/46,XX mosaicism and minor karyotype variants were not included. Testing with oral glucose tolerance test (OGTT) also allowed an estimate of occult glucose intolerance in women with TS. Metabolic status in women with established DM was made on a fasting blood sample for insulin glucose from which insulin resistance could be estimated using HOMA-IR. C-peptide was used as a measure of insulin secretory capacity. Lastly, diabetes related autoimmunity was assessed in all sample.

Characterisation of glucose homeostasis and diabetes mellitus in adults with Turner Syndrome

Table 6.1 is a descriptive table before statistical analysis showing the OGTT outcomes. The data from those with IGT was combined with that of newly diagnosed DM women to represent those whom newly abnormal glucose metabolism. OGTT were conducted in 76 women, added to this was one historical OGTT. Final sample size was 77 from the following karyotype backgrounds; 45,X (48.4%); 45,X/46,X,r(X) (27.1%);45,X,i(X) & 45,X/46,X,i(X) (24.5%). As defined by the UCLH OGTT protocol; 15/77 (19.5%) were diagnosed as having IGT and

6/77 (7.6%) were newly diagnosed as T2DM. The remaining individuals 56 individuals (72.7%) were classified as Normal Glucose Tolerance (NGT).

Of the established diabetic subjects (n= 29), one participant was reported as having adult onset T1DM, 3.4%. The remaining subjects were reported to have T2DM, 96.6%. Average age of diagnosis was 36.1 years (range 11- 56). Figure 6.1 shows the cumulative frequency of the age of onset those with new and established TS-associated DM. For illustration, estimated age of onset of T1DM and T2DM is plotted alongside as a means of comparison. Figure 6.1 demonstrates how the age of TS-associated DM onset lies between the two references ranges for T1DM and T2DM. Mean duration of DM was 9.1 years (range 7 months – 25years, ± 5.5 years). DM treatment was reported in all 29 women. Four treatment groups were identified; 21/29 (72.4%) oral antidiabetics, 5/29 (17.2%) insulin therapy, 2/29 (6.9%) dietary intervention, 1/29 (3.3%) insulin and oral anti-diabetics combined.

Table 6.1 descriptive table showing the parameters investigated as part of the Turner Syndrome-associated Diabetes mellitus (DM) characterisation for each of the subgroups studied as part of study 3 before statistical analysis. The standard deviation is presented in brackets for anthropometric measures and interquartile range (IQR) for biochemical parameters.

	Oestrogen Deficient Reference Range	Normal glucose tolerance	Impaired Glucose Tolerance/ newly diagnosed DM	Established DM
N	15	56	21	29
Age	31 (± 11.1)	32.1 (± 10)	31.3 (± 7.8)	44.5 (± 11.8)
Anthropometric				
BMI	24 (± 6.2)	25.6 (± 6.2)	30.6 (± 7.3)	29.7 (± 6.8)
Waist Circumference (cm)	83.9 (± 20.6)	84.1 (± 13.4)	92.4 (± 19.9)	98 (± 13.9)
Body Fat %	31.2 (± 9.6)	27.9 (± 11.2)	35 (± 11.3)	31.7 (± 11.3)
Biochemical				
Fasting glucose	4.5 (4.4 – 4.8)	4.4 (4.2 – 4.7)	4.8 (4.4 – 5.5)	7.4 (5.0-9.8)
Fasting insulin	8.2 (6.4 – 10.7)	5.5 (4.1 – 7.2)	6.0 (3.7 – 9.1)	8.0 (3.6 – 12.2)
C-peptide	506 (453 – 566)	422 (322.8 – 533)	718 (509 – 1075.8)	679 (437 – 1317)
HbAc1	5.3 (5 – 5.4)	5.2 (4.9 – 5.5)	5.4 (5.1 – 5.8)	7.9 (6.1 – 8.6)
HOMA-IR	1.8 (1.4 – 2.1)	1.1 (0.8 - 1.4)	1.3 (0.7 – 2.1)	2.0 (1.2 – 5.2)
Autoimmunity				
TPO+	7.7%	43.4%	35%	46.4%
GAD+	0%	3.8%	0%	22.6%
IA-2+	0%	3.8%	7.7%	7.4%
ZnT8+	0%	0%	0%	4.7%

Figure 6.1 the graph shows the age of onset for Turner Syndrome-associated Diabetes mellitus and includes newly diagnosed cases as part of study 3. Included is the published data on the age of onset of those with T1DM and T2DM as a means of comparison.

Cumulative frequency of age of onset Turner Syndrome-associated DM compared to that of T1DM & T2DM
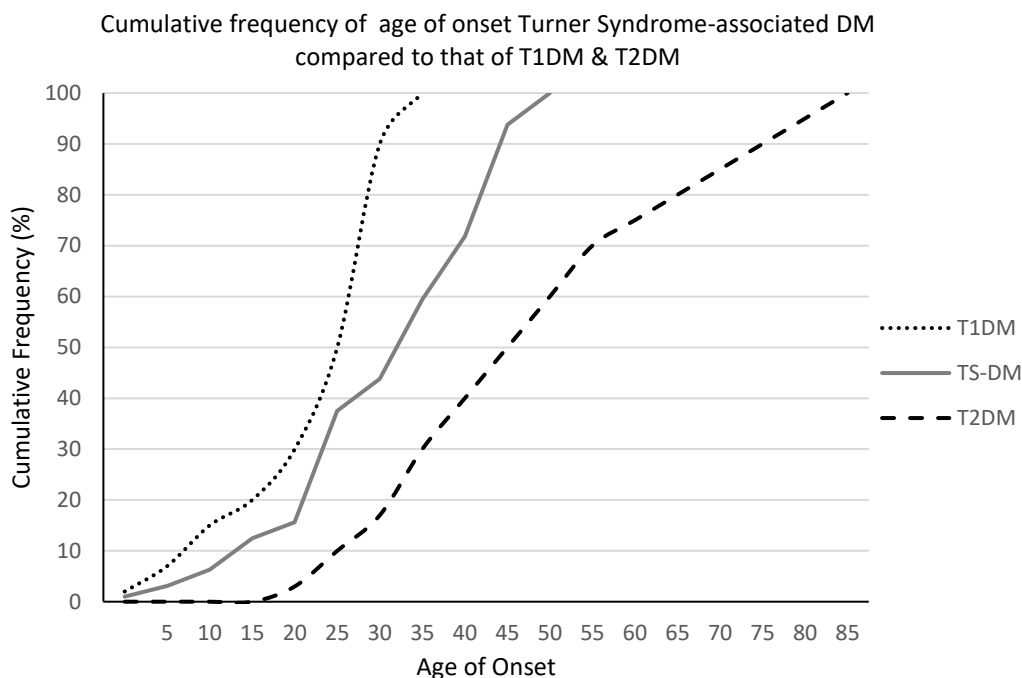
Relationships between karyotype & glucose metabolism

UCLH records were used to identify women from karyotype backgrounds of interest. Karyotype data was available 98/106 subjects (92.5%). The remaining 9 either had no karyotype information available or were DM-subjects from non-relevant groups.

The recruited women represented the following percentage of the total number of those on record; 45,X 22.7%; 45,X/46,X,r(X) 45.8%; 45,X,(iX) 60.5% (table 6.2). Chi-squared analysis was performed comparing the prevalence of IGT/DM from those with the ring chromosome and isochromosome to that of 45,X separately. The prevalence those individuals affected by IGT/DM did not differ to that of 45,X in either the ring chromosome or isochromosome.

Table 6.2 shows the prevalence of Normal Glucose Tolerance (NGT) versus Impaired Glucose Tolerance/ Diabetes mellitus (IGT/DM) for the ring chromosome and isochromosome karyotype groups compared to that of 45,X.

	45,X	**45,X/46,X,r(X)**	**45,X,i(X)**
UCLH Records	270	48	28
N	53	22	17
NGT	52.8%	68.2%	70.6%
IGT/DM	47.2%	31.8%	29.4%

Glucose homeostasis in Turner Syndrome-associated diabetes mellitus

Table 6.3 summarises the key DM-associated parameters investigated as part of study 3. Those with NGT were compared to the oestrogen deficient reference range and IGT/DM separately.

No significant differences were found between the oestrogen deficient reference range and NGT women with TS with regards to age, anthropometric measure such as BMI, c-peptide and glucose related parameters. The same relationship was not found for insulin related parameters; fasting insulin and HOMA-IR (table 6.3). Fasting insulin was significantly higher in those with POI versus NGT (8.2 mmol/L versus 5.5 mmol/L, p= ≤0.01). Due to the insulin based natured of HOMA-IR, HOMA-IR was significantly higher in those with POI (1.8 versus 1.1, p= ≤0.01).

The finding of higher fasting insulin among women with POI when compared to NGT women was unexpected. Given the smaller sample size of the POI group (n= 15) it is possible a sampling error may have occurred. Evidence for which is seen in the c-peptide measurement as c-peptide, the precursor to insulin, did not significantly differ between POI control and NGT women (table 6.3).

For the purposes of analysis IGT and DM groups were combined. IGT/DM subjects were found to be significantly older when compared to NGT subjects (p= ≤0.01) As expected, glucose related parameters such as HbAc1 and fasting glucose were also significantly higher within the IGT/DM (p= ≤0.01).

Insulin resistance is defined by a calculated HOMA-IR ≥2.5. Diabetic and IGT subjects were found to have a higher degree of insulin resistance when compared to the NGT group (median 1.5 IQR 0.8 – 3.6 versus median 1.1 IQR 0.8 – 1.4, p= ≤0.01). Fasting insulin followed the same association (p= ≤0.01). As expected from these findings, c-peptide was significantly higher within the IGT/DM group indicating a transition to a state of insulin resistance (IR) (table 6.3). In descriptive table 6.2, shows the transition to IR; IGT and newly diagnosed have a greater concentration of c-peptide whilst NGT subjects had the lowest levels (median 422 pmol/L IQR 33.8 - 533 versus 669 IQR 437 - 1317 pmol/L). Established diabetics were found to have c-peptide levels between those of NGT and IGT/ newly diagnosed DM group largely representing the effects of treatments such as metformin (table 6.2).

The vast majority of diabetics in the UCLH cohort take oral anti-diabetics to control blood glucose. However, some subjects have a poor response to oral medication and therefore, may switch to insulin therapy without an assessment of beta-cell function in the first instance. In a sub-analysis of DM subjects, those who treated their diabetes using metformin had a significantly higher levels of c-peptide when compared to those taking insulin (median 736 pmol/L IQR 568 – 1582.8 versus 39 pmol/L IQR 33 – 236.3, p= ≤0.001).

Associated with the T2DM phenotype is an increased BMI, fat mass and waist circumference (Feller, Boeing, & Pischon, 2010; Solanki, Makwana, Mehta, Gokhale, & Shah, 2015). In the UCLH IGT/DM series, waist circumference and BMI were higher when NGT subjects (p= ≤0.001 respectively, table 6.3). Which also translated into a higher body fat percentage than NGT subjects (33.2% versus 28.2%, p= 0.03, table 6.3).

Table 6.3 shows the parameters investigated as part of the Turner Syndrome-associated diabetes mellitus characterisation. Included are; mean anthropometric measures, median biochemical outcomes and percentage of positive autoantibodies. Those with IGT and DM cases have been combined for the purpose of analysis and were individually compared to NGT subjects. The standard deviation is presented in brackets for anthropometric measures and interquartile range (IQR) for biochemical parameters. Superscript ^{A} represents the NGT group with * denoting a p= <0.05 and ** denoting p= ≤0.01.

	Oestrogen Deficient Reference Range	NGT	IGT/DM
N	15	56	50
Age	31 (± 11.1)	32.1 (± 10)	39.3 (± 12.4)^{A**}
Anthropometric			
BMI	24 (± 6.2)	25.6 (± 6.2)	29.7 (± 6.6)^{A**}
Waist Circumference (cm)	83.9 (± 20.6)	84.1 (±13.4)	95.6 (± 15.4)^{A**}
Body Fat %	31.2 (± 9.6)	27.9 (± 11.2)	33.2 (± 9.6) ^{A*}
Biochemical			
Fasting glucose	4.5 (4.4 – 4.8)	4.4 (4.2 – 4.7)	5.4 (4.5 – 7.4) ^{A**}
Fasting insulin	8.2 (6.4 – 10.7) ^{A**}	5.5 (4.1 – 7.2)	7.2 (3.6 – 10.6) ^{A**}
C-peptide	506 (453 – 566)	422 (322.8 – 533)	682 (450 - 1266) ^{A**}
HbAc1	5.3 (5 – 5.4)	5.2 (4.9 – 5.5)	5.8 (5.3 – 8) ^{A**}
HOMA-IR	1.8 (1.4 – 2.1) ^{A**}	1.1 (0.8- 1.4)	1.5 (0.8 – 3.6) ^{A**}
Autoimmunity			
TPO+	7.7%^{A*}	42.5%	42.5%
GAD+	0%	3.8%	15.9% ^{A*}
IA-2+	0%	3.8%	9.4%
ZnT8+	0%	3.9%	2.3%

Autoimmunity in Turner Syndrome related to diabetes mellitus

Table 6.4 shows that DM related autoantibodies GAD, IA-2 and ZnT8 were not present in POI (0/15 compared to 9/106, p= 0.02). In women with TS, IA-2 and ZnT8 were rarely positive (5/106 and 1/106 respectively) and not significantly different from POI or between diabetes group (table 6.3). There was a trend to GAD positivity being more common in women with IGT/DM (p= 0.07) (table 6.3). When examining autoantibody status within TS, those with IGT/DM were found to have a trending higher rate of GAD positivity than NGT (15.9% versus 3.8%. p= 0.058). Notably, 3/5 DM subjects who used insulin therapy were GAD positive compared to 3/17 subjects who took metformin or other oral anti-diabetics (p= ≤0.01).

In the general population positive GAD autoantibodies are associated with poor insulin secretion and an earlier age of DM onset (Hagopian et al., 1993) (Leslie, Palmer, Schloot, & Lernmark, 2016). Table 6.4 shows insulin related parameters in relation to GAD autoantibody status. There was no statistical difference between GAD positivity and insulin parameters or age of DM onset. However, a trend can be a seen whereby those who are GAD positive have a lower c-peptide concentration and consequently lower fasting insulin and calculated HOMA-IR. A subsequent ANOVA analysis of those with established DM with GAD autoantibodies saw c-peptide concentrations to be significantly reduced (201.1 pmol/L versus 1010.4 pmol/L, p= ≤0.01) with a trending lower fasting insulin (4.2 pmol/L versus 11.3 pmol/L, p= 0.06).

Table 6.4 shows the insulin related parameters against GAD autoantibody status. Included is the age of onset of diabetes mellitus for those with DM.

	GAD-	GAD+
Age of DM Onset	34.9 (± 12.2)	37.3 (± 8.2)
Fasting Insulin	8.2 (± 5.4)	5.0 (± 3.9)
C-peptide	785.7 (± 831.9)	437.7(± 286.6)
HOMA-IR	1.9 (± 1.9)	1.2 (± 1)

The isochromosome has previously been associated with an excess risk of autoimmunity (Gawlik et al., 2018; Grossi et al., 2013). Women with 45,X,i(X) were selected for analysis against those with 45,X and 45,X/46,r(X). In study 1, no associations were found between the isochromosome and autoimmunity. A further, 6 women with mosaic isochromosome were included within the 45,X,iX group for analysis. No excess autoimmunity was found.

Hypothyroidism and TPO antibodies were included in this assessment because of the observation in study 1 of an association between hypothyroidism and existing diabetes. Also, TPO may be less prone to decline over time compared to GAD antibodies. The presence of positive TPO was higher in the TS when compared to the POI controls (p= 0.01). However, there was no difference between the presence of TPO positive antibodies between TS groups. There was no difference in the prevalence of hypothyroidism between all groups.

Estimating risk of T2DM in women with Turner Syndrome

OGTT identified IGT in 19.5% and T2DM 7.8% of the those tested. ROC modelling was used to identify if parameters used in the general population to estimate DM-risk were applicable to women with TS. ROC modelling of BMI, waist circumference, HbAc1 and fasting glucose was performed in 76 women submitted for an OGTT. Data from those with established DM was excluded as women were currently undertaking treatment or lifestyle modifications. In this analysis, fasting glucose and HbAc1 were found to be the least sensitive predictors of DM (AUC= .7, p= 0.02 and AUC= 0.73, p= 0.01 respectively). The

most accurate of the 4 parameters of DM was found to be waist circumference and BMI, (AUC= 0.77, p= 0.02 respectively). Evidence for which can be found in table 6.3 as those IGT and newly established DM were found to have similar fasting glucose levels to those with NGT after a 120' minute OGTT.

BMI is a well-established measure collected in TS-clinics. The BMI ROC curve was used to establish a BMI cut-off to triage women with TS for OGTT (Figure 6.2). The black line was used to identify the optimal sensitivity against specificity, (0.7 sensitivity and a specificity of 0.22). The coordinates were then used to identify a BMI cut-off of 27.

Risk of diabetes is also related to ethnicity and family history. Ethnicity was recorded for 97/107 (90.7%). Broadly, ethnicity was categorised into three 4 main groups; white-European (72.4%), African descent (11.3%), South Asian (9.3%) and other (5.2%), which included people who were from bi-racial backgrounds or did not fit into the three other categories. The distribution of IGT/DM cases did not significantly differ to that of the NGT group.

Participants were asked to report whether a first-degree relative was affected by T2DM. There was no association between a positive family history and DM status in all instances.

Figure 6.2 plot of the Receiver Operator Characteristic (ROC) analysis of BMI produced by SPSS. The red line indicates the ROC model. The blue line represents the BMI data plotted for 76 women who were submitted for an OGTT. The black line was used to identify the optimal sensitivity against specificity
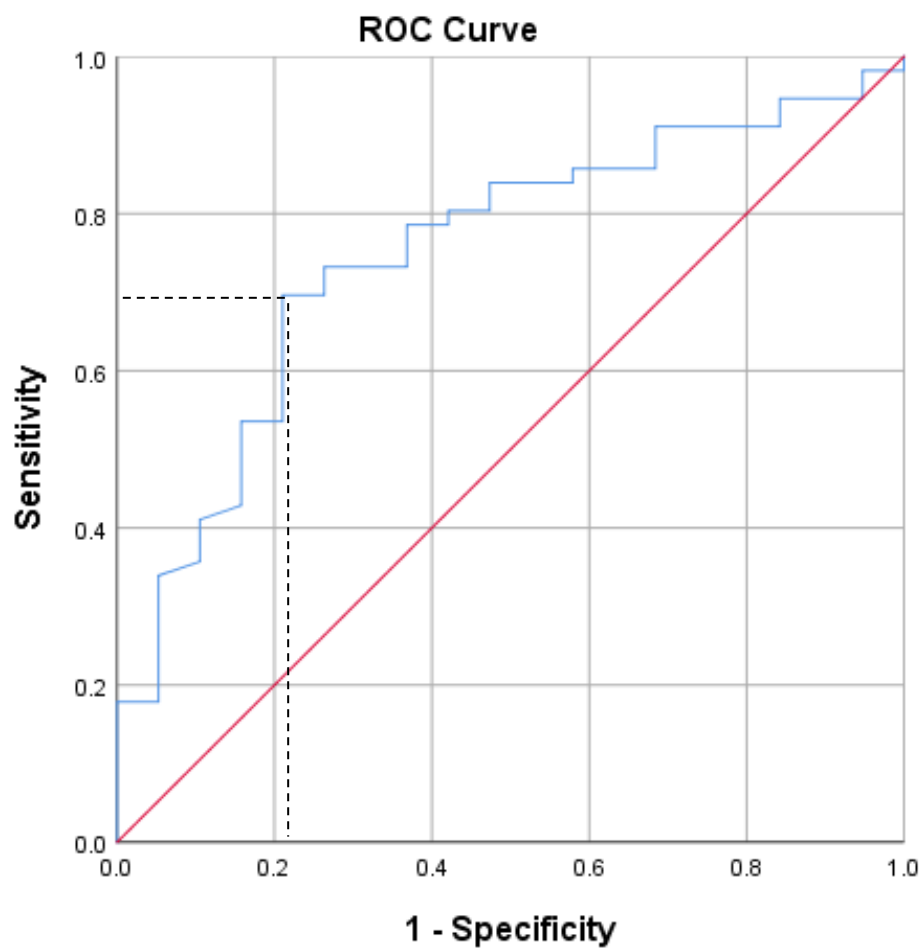
Chapter VII

Pilot genetic characterisation of common variants associated with T2DM in women with Turner Syndrome

The aim of this study was to explore the possibility that the genetic influence of diabetes risk in women with TS may by determined by an interaction between loss of X chromosome material with known T2DM associated SNPs. A literature review conducted as part of chapter I found just two potential T2DM X-linked candidate gene *INSR4* and *FOXP3.*

To date, more than 146 risk variants have been shown to be associated with T2DM (Xue et al., 2018) and I narrowed down a list for a pilot study based on frequency of the risk allele (reported Minor Allele Frequency ≥20%). The result was a list of a list of 19 key T2DM SNPs.

Methodology

Samples

The samples submitted for GWAS were those obtained from consenting individuals from study 3. Additional samples were also obtained for the purpose of this sub-study. These individuals had no previous history of IGT or DM as well as no history of an elevated HbAc1. This larger group consisted of 92 controls and 45 cases (n= 137).

Infinium global screening array v3.0

The Ilumina Infinium Global Screening Array v3.0 is a Beadchip microarray. Beadchip technology uses silica beads covered with thousands of copies of specific oligonucleotides. The oligonucleotides capture complementary DNA sequences when exposed to complementary DNA. Some 654,027 oligonucleotides are found on the array, from the 26 Genome Project populations and cover the whole genome.

Two key qualities of the Ilumina Infinium Global Screening Array v3.0 make it ideal for application in clinical research or in specific disease state populations. Firstly, the array is multi-ethnic, meaning its application in populations obtained from a clinical setting or in specific disease states where subjects may be recruited from varying ancestral backgrounds is highly useful. Secondly, the curated SNPs are derived from human variation reporting databases such as ClinVar, CPIC, and PharmGKB. Therefore, the SNPs are highly clinically relevant.

Literature search for known T2DM variants

The *Nature* journal publication search tool was used to identify diabetes meta-analysis conducted during 2018 - 2019. Studies which used meta-analysis were selected for, given their power. Of 107 publications, 2 paper met the search parameters.

An individual search was then conducted within each of the two papers for known common T2DM SNP's. Common SNPs was defined as SNPs occurring with a Minor Allele Frequency (MAF) ≥20%. SNPs occurring with a MAF less than 20% were deemed too rare. Whilst novel SNPs were excluded as reproducibility is not yet known. Finally identified SNPs were then checked for their presence on the Ilumina Infinium Global Screening Array v3.0

Mahajan et al., conducted an expanded meta-analysis GWAS study combining data from approximately 900,000 Europeans to produce the largest study of its kind to date (Mahajan et al., 2018). A total of 218 previously reported SNPs was reproduced by the study (Mahajan et al., 2018). A review of the 218 SNPs was conducted using the above criteria.

The second paper was by Flannick et al., (2019). The multi-ancestry exome sequencing study of 20,791 diabetic cases versus 24,440 controls. Of which 10,517 were of European descent. Fifty variants achieved exome-wide significance (Flannick et al., 2019). However, only 9/50 achieved significance within the European population. The 9 SNPs were specifically not quoted. A review of the 50 SNPs was conducted using the above criteria.

The identified SNPs were then ranked in terms of MAF and Odds Ratio (OR). A minimum OR ≥1.05 cut-off was arbitrarily set as variants with an OR above 1.05

were assumed to be more informative. The result was a list of 19 key T2DM-associated SNPs.

DNA extraction & quantification

DNA extraction

DNA was extracted from frozen whole blood using Qiagen QIAamp DNA Blood Mix protocol for 5ml of whole blood (*see appendix for protocols*). In some instances, patients obtaining a full 5ml EDTA tube of blood was not possible and therefore the Qiagen QIAamp DNA Blood Mix protocol for 3ml was implemented for extraction. After health and safety measures were used including the use of two sets of gloves, goggles and all extractions were performed under a fume hood. The Qiagen QIAamp DNA Blood Mix Midi and Maxi protocols follow the same process tailored to the volume of blood.

Blood samples were removed from the -20°C freezer and placed within a 37°C water bath for 5 minutes until the blood had thawed. After the samples had thawed protease and buffer solution are added to break open lymphocytes and digest unwanted proteins. The solution was incubated a 70°C, and ethanol is added to the solution. Ethanol creates hydrogen bonds with water decreasing the amount soluble DNA thus increasing DNA concentration and also serves to remove contaminants from the earlier stages. The solution is then transferred to the Qiagen Maxi or Midi Column. A series of centrifugations, buffer washes and incubations followed. During these stages the extracted DNA is captured on the material located within the column as filtrates pass through the column. The final stage is marked by using nuclease-free water to elute DNA followed by room temperature incubation and centrifugation. To maximise DNA yield, the elution stage was repeated twice using the elute from the first centrifugation. An initial DNA quantification was performed in order to assess the success of the DNA extraction using Thermo Fisher Scientific NanoDrop. If a sample was noted to have a low yield, ≤30ng the step spin step and nanodrop repeated before freezing at -20°C.

DNA quantification and lyophilisation

Extracted DNA was thawed from the -20°C freezer for quantification, before library preparation, by Qubit Fluorometer. Extracted DNA samples, 1 µl, were mixed with the provided Qubit Fluorometer dye. The dye binds to the DNA enabling it to fluoresce. High and low concentration reference samples, provided by Qubit, are then used to establish a reference range for which the samples of interest are measured against.

Optimum DNA concentration for the Infinium Global Screening Array v3.0 is between 50 - 150ng. The concentration of some samples had reduced during their time in storage and therefore required lyophilisation. Lyophilisation, is a process by which extracted samples are placed in a heated centrifuged. Once initiated, the heated centrifuge evaporates excess water from the samples and thus increases DNA concentration. Further lyophilisation was conducted at 10-minute intervals and the concentration remeasured until all samples were brought into the optimum DNA concentration range for the array platform.

DNA library preparation using Illumina NGS STAR Hamilton

Library preparation was conducted using an automated platform NGS STAR Hamilton at UCL genomics (*see appendix for protocol*). In summary, DNA was amplified and incubated. DNA was then fragmented and a series of precipitation and resuspension steps follow before the DNA is exposed to the Illumina Beadchip. For more details regarding Beadchip technology please see Study 4 methodology: Infinium Global Screening Array v3.0.

Statistics

Genotype data requires a series of steps; power calculations, genotype control, genotype modelling to assess risk allele penetrance, measure of the effect of SNP and tests of association. To account for multiple testing the p value was reduced to 0.01 to indicate significance.

Pilot study power calculation

As part of the initial workflow for study 4, a power calculation was performed by the Institute of Child Health UCL. The power calculation was used to determine the optimal cohort size given that IGT/DM occurs in around 20%. It was calculated

that for study 4 a ratio of two controls to every IGT/DM case should be used,
Figure 7.1 optimal cohort size to detect variants at a range of powers and deltas.
Delta, plotted along the y axis, in this instance represents the size of the
difference between case-control populations. Cohort size and delta values has
an inverse relationship; as cohort size increases, delta reduces as cohort smaller
differences can be detected. Figure 7.1 shows that at the minimum power, 0.8
(red line) at least 100 individuals are needed in order to detect large significant
differences between cases and controls.

Figure 7.1 is a plot of power calculation performed by the Institute of Child Health.
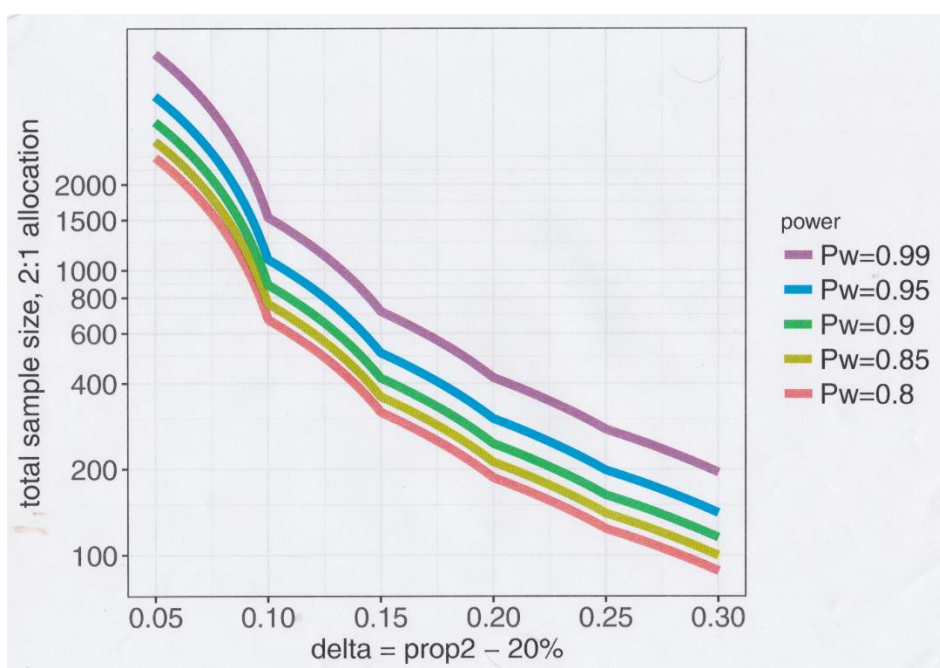
Genotyping quality control

Hardy Weinberg Equilibrium (HWE) is a common statistical test used for quality
control in population array-based studies. HWE assumes that there is; no
mutation, selection, migration or genetic drift in large stable populations and
therefore genotype frequencies become a function of allele frequencies
(Namipashaki, Razaghi-Moghadam, & Ansari-Pour, 2015). Deviation from HWE
may suggest any of the following events has occurred; selection bias, population
stratification or genotyping errors (Namipashaki et al., 2015). HWE is assessed
by a chi squared goodness-of-fit test (critical value 5.99 with 1 degree of

freedom). Although it is not necessary for HWE to be true for case-control studies, as cases are selected non-randomly, HWE was conducted within the control group for the purpose of identifying genotyping error (Namipashaki et al., 2015).

Genotype & allele modelling

Case-control population-based study designs compared the frequency of genotypes or alleles. Models can be informative of the penetrance of the risk allele within the population (Clarke et al., 2011; Lewis, 2002). The standard models used are; dominant, recessive and multiplicative. The dominant and recessive models are based on genotype frequency whilst the multiplicative is based on allele frequencies (Clarke et al., 2011; Lewis, 2002). Under the dominant model both the risk allele heterozygous and heterozygous genotypes are combined to be compared to genotype frequencies of the recessive. Whilst under the recessive model the risk recessive genotype is compared to the non-risk and heterozygous genotype combined (Clarke et al., 2011; Lewis, 2002). The multiplicative model states there is an increase in risk with each additional risk allele. Chi-squared or Fisher's exact tested whether the distribution of allele or genotypes differed between cases and controls.

Measure of SNP effect

Odds Ratio (OR) in population-based studies are used to measure the effect size of a SNP. OR uses allele counts of the risk allele in the IGT/DM group versus controls (Thomas, Suresh, & Suresh, 2013). The result is the probability of an event occurring, in this case T2DM. An OR probability closer to 1 would indicate no association. Whilst a OR≥1 would indicate the risk allele increases disease probability (Thomas et al., 2013).

Case-control SNP association

Linear regression analysis was performed to test for SNP association with case-control status. Three regression analyses were performed using differing genotype models. Genotypes were portioned in order to increase power using models like those used in the genotype and allele modelling portion of the study (*see Statistics relating to study 4: Genotype and Allele Modelling*). With the only exception that heterozygous genotypes were tested against all other groups separately. T2DM is known to be influenced by both age and BMI of subjects and

therefore these factors were adjusted for in line with previous similar works (Mahajan et al., 2018).

Results: general

A total number of 137 individuals (45 IGT/DM cases versus 92 controls) were submitted for genotyping across 19 known T2DM SNP. The SNPs were reported to have an MAF ≥20% (table 7.1).

Table 7.1 displays the 19 T2DM-associated SNPs identified as a result of a literature review. SNPs were chosen based on a 1) Minor Allele Frequency above 20% 2) an Odds Ratio (OR) above 1.05 3) presence on the Infinium Global Screening Array v3.0 (Mahajan et al., 2018; Flannick et al., 2019).

SNP number	Gene	Position Build 37	MAF (%)	Reference OR
rs1046314	WSF1	4:6303955	49	1.1 (1.06-1.14)
rs1046317	WSF1	4:6304242	32	1.1 (1.06-1.14)
rs10830963	MTNR1B	11:92708710	27.7	1.10 (1.09-1.12)
rs11257655	CDC123/CCMK1D	10:12307894	21.8	1.09 (1.08-1.11)
rs11708067	ADCY5	3:123065778	22.8	1.09 (1.08-1.11)
rs11926707	KIF9	3:46925539	37.4	1.27 (1.17-1.38)
rs1260326	GCKR	2:27730940	39.3	1.07 (1.06-1.08)
rs13266634	SLC30C8	8:118184783	43	1.16 (1.11-1.22)
rs1359790	SPRY2	13:80717156	28	1.09 (1.07-1.10)
rs1421085	FTO	16:53800954	41.5	1.13 (1.12-1.15)
rs2237895	KCNQ1	11:2857194	42.6	1.12 (1.11-1.14)
rs3768321	MACF1	1:40035928	20	1.09 (1.07-1.10)
rs3802177	SLC30C8	8:118185025	31.5	1.11 (1.10-1.13)
rs5215	KCNJ11	11:17408630	39	1.08 (1.05-1.12)
rs5219	KCNJ11	11:17409572	39	1.07 (1.04-1.1)
rs757110	ABCC8	11:17418477	39	1.07 (1.05-1.1)
rs7756992	CDKAL1	6:20679709	27.4	1.15 (1.13-1.17)
rs7903146	TCF7L2	10:114758349	29.5	1.37 (1.35-1.39)
rs2290854	MDM4	1:204516025	72	1.1 (0.97-1.05)

Table 7.1 shows the final set of T2DM-associated SNPs which were genotyped using the Illumina Infinium Global Screening Array v3.0 along with the nearest gene, allele frequency and reference OR. All SNPs were genotyped successfully except in the case of rs1046314, where genotyping failed for one individual (n= 136).

Table 7.2 summarises the quality controls outcomes. HWE was calculated within the NGT/ no history group with a HWE critical value <5.99 indicating no significant difference between observed and expected genotypes. All SNPs were found to be in HWE. Suggesting there was no evidence of genotyping error or departure from observed and expected genotypes (table 7.2). A second measure of quality control was undertaken by comparing MAF of both cases and controls to that of reference database GnomAd non-Finnish population data (table 7.3). The risk allele was taken from that the previously published literature (Flannick et al., 2019; Mahajan et al., 2018). There were no obvious differences between the TS-population allele frequency and that of the reference data set. (table 7.2).

OR were calculated using TS-population allele frequency data (table 7.2). As all SNPs genotyped had been previously identified for their involvement in T2DM previous OR values were reported to be ≥1. However as seen in OR comparison table 7.3, many of the calculated OR values in the TS population were lower than that published with wide confidence intervals (CI). Some SNPs that did achieve an OR above 1 did not achieve significance as a result of the wider confidence intervals. Except in the case of rs1359790 (p= 0.01) which achieved significance and rs10830963 were a trend was noted (p= 0.03) (*see SNP specific results for more detail*).

Genotype and allele models were used to examine risk allele penetrance. Table 7.2 shows the results of the multiplicative model which uses allele frequency and therefore has an increased power. Under the or multiplicative model one SNP was found to be significant; rs1359790 (OR 0.3, p= ≤0.01) whilst rs10830963 trended (OR 1.9, p= 0.04) (*see SNP specific results for more detail*).

Linear regression analysis was performed for each SNP against between case-control-status under three different genotype models; recessive, dominant and heterozygous (table 7.4). The regression was performed both unadjusted then adjusted for BMI and age, known influential factors in T2DM.

Generally, BMI was found to be the most influential factor in T2DM under the linear regression. The SNP-specific results of the linear regression can be found in table 7.4. Two SNPs were found to positively trend rs10830963 (dominant model, p= 0.056) and rs1046317 (p= 0.051, heterozygous model) with IGT/DM status. When adjusted for BMI and age rs1046317 lost significance. Similarly, under the unadjusted linear regression rs1046314 had a positive association under the heterozygous model (p= 0.006) which when adjusted for BMI and age resulted in a trend (p= 0.058). Finally, rs1359790 (dominant model, p= 0.014) and rs7903146 (heterozygous model, p= 0.002) were found to be significant under the adjusted models. However, rs7903146 was found to have a negative association when adjusted for age and BMI (*see SNP specific results for more detail*).

Table 7.2 shows each of the 19 T2DM SNPs genotyped against the GnomAd non-Finnish European allele frequency. Included are also the allelic modelling p-value for multiplicative model and calculated odds ratio as well as the control group hardy-Weinberg equilibrium calculation. * denotes a p-value ≤0.01. (Mahajan et al., 2018; Flannick et al., 2019)

	GnomAd Reference Variation	Risk Allele	GnomAd non-Finnish European allele frequency		TS Impaired Glucose Tolerance & Diabetics (n= 45)		TS NGT/ no previous history glucose impairment (n= 92)		NGT HWE X^2 (<5.99)	Allele Modelling Multiplicative Model p-value	OR (95% CI)	OR (95% CI) P value
			Ref.	Alt.	Ref.	Alt.	Ref.	Alt.				
rs1046314	G>A	A	0.41	0.59	0.44	0.56	0.42	0.58	3.1	0.7	0.9 (0.6-1.5)	0.7
rs1046317	T>C	C	0.33	0.67	0.28	0.72	0.31	0.69	0.8	0.6	1.2 (0.7-2)	0.6
rs10830963	C>G	G	0.71	0.29	0.7	0.3	0.81	0.19	0.2	0.04	1.9 (1-3.4)	0.03
rs11257655	C>T	T	0.80	0.20	0.81	0.19	0.83	0.17	0.02	0.8	1.1 (0.6-2.1)	0.8
rs11708067	A>G	A	0.79	0.21	0.79	0.21	0.83	0.17	0.1	0.4	0.8 (0.4-1.4)	0.4
rs11926707	T>C	C	0.38	0.62	0.5	0.5	0.42	0.58	0.4	0.2	0.7 (0.4-1.2)	0.2
rs1260326	T>C	C	0.41	0.59	0.47	0.53	0.37	0.63	1.3	0.1	0.6 (0.4-1.1)	0.1
rs13266634	C>T	C	0.70	0.30	0.8	0.2	0.73	0.27	0.07	0.2	1.3 (0.8-2.7)	0.2
rs1359790	C>T	C	0.72	0.28	0.66	0.34	0.78	0.22	1	0.02	0.3 (0.2-0.5)	<0.01*
rs1421085	T>C	C	0.57	0.43	0.62	0.38	0.63	0.37	5.9	1	1.2 (0.7-2.1)	0.5
rs2237895	A>C	C	0.58	0.42	0.56	0.44	0.54	0.46	3.4	0.8	0.9 (0.6-1.5)	0.8
rs3768321	C>T	T	0.80	0.2	0.83	0.17	0.84	0.16	0.2	0.4	1 (0.5-2)	0.9
rs3802177	C>T	C	0.68	0.32	0.8	0.2	0.73	0.27	0.01	0.2	1.5 (0.8-2.8)	0.2
rs5215	C>T	T	0.37	0.63	0.28	0.72	0.35	0.65	0.5	0.3	1.4 (0.8-2.4)	0.3
rs5219	T>C	T	0.37	0.63	0.28	0.72	0.34	0.66	0.04	0.2	0.8 (0.4-1.3)	0.3
rs757110	G>T	T	0.37	0.63	0.3	0.7	0.34	0.66	0.04	0.5	0.8 (0.5-1.5)	0.5
rs7756992	A>G	G	0.71	0.29	0.67	0.33	0.65	0.35	0.5	0.7	0.9 (0.5-1.6)	0.7
rs7903146	C>T	T	0.68	0.32	0.66	0.34	0.72	0.28	0.7	0.3	1.3 (0.8-2.3)	0.3
rs2290854	A>G	A	0.30	0.70	0.62	0.38	0.62	0.38	0.2	0.5	1.0 (0.6-1.7)	1

Table 7.3 shows the calculated Odds Ratio (OR) for the 19 T2DM SNPs genotyped as part of the pilot case-control study. As a means of comparison, the OR from the reference's studies along with the SNP significance is also displayed (Mahajan et al., 2018; Flannick et al., 2019)

SNP	OR (95% CI)	P value	Reference Odds Ratio	Reference P value
rs1046314	0.9 (0.6-1.5)	0.7	1.1 (1.06-1.14)	1.30×10^{-06}
rs1046317	1.2 (0.7-2)	0.6	1.1 (1.06-1.14)	1.30×10^{-06}
rs10830963	1.9 (1-3.4)	0.03	1.10 (1.09-1.12)	4.8×10^{-43}
rs11257655	1.1 (0.6-2.1)	0.8	1.09 (1.08-1.11)	1.5×10^{-32}
rs11708067	0.8 (0.4-1.4)	0.4	1.09 (1.08-1.11)	5.2×10^{-32}
rs11926707	0.7 (0.4-1.2)	0.2	1.27 (1.17-1.38)	2.1×10^{-8}
rs1260326	0.6 (0.4-1.1)	0.1	1.07 (1.06-1.08)	6.5×10^{-25}
rs13266634	1.3 (0.8-2.7)	0.2	1.16 (1.11-1.22)	2.2×10^{-11}
rs1359790	0.3 (0.2-0.5)	<0.01*	1.09 (1.07-1.10)	2.4×10^{-31}
rs1421085	1.2 (0.7-2.1)	0.5	1.13 (1.12-1.15)	3.1×10^{-84}
rs2237895	0.9 (0.6-1.5)	0.8	1.12 (1.11-1.14)	6.0×10^{-52}
rs3768321	1 (0.5-2)	0.9	1.09 (1.07-1.10)	2.6×10^{-26}
rs3802177	1.5 (0.8-2.8)	0.2	1.11 (1.10-1.13)	1.1×10^{-55}
rs5215	1.4 (0.8-2.4)	0.3	1.08 (1.05-1.12)	4.4×10^{-06}
rs5219	0.8 (0.4-1.3)	0.3	1.07 (1.04-1.1)	6.7×10^{-06}
rs757110	0.8 (0.5-1.5)	0.5	1.07 (1.05-1.1)	5.0×10^{-09}
rs7756992	0.9 (0.5-1.6)	0.7	1.15 (1.13-1.17)	2.4×10^{-88}
rs7903146	1.3 (0.8-2.3)	0.3	1.37 (1.35-1.39)	5.8×10^{-447}
rs2290854	1.0 (0.6-1.7)	1	1.1 (0.97-1.05)	4.5×10^{-06}

Table 7.4 displays for the for the result of the linear regression analysis for each of the 19 T2DM SNPs genotyped. Shown is the penetrance model used; recessive (risk genotype compared to all others, heterozygous model (heterozygous genotype compared to all others) and dominant model (risk and heterozygous genotype s compared to non-risk genotype). Included is the β-correlation unadjusted and again adjusted for BMI and age. * indicates those SNPS which DM status ≤0.01.

SNP	Recessive Model				Heterozygous Model				Dominant Model			
	Unadjusted	P Value	Adjusted BMI & Age	P Value	Unadjusted	P Value	Adjusted BMI & Age	P Value	Unadjusted	P Value	Adjusted BMI & Age	P Value
rs1046314	0.98	0.26	0.6	0.478	0.237*	0.006*	0.163	0.058	-0.195	0.024	-0.131	0.121
rs1046317	0.11	0.203	0.066	0.429	-0.168	0.051	-0.097	0.256	-0.094	0.276	-0.04	0.634
rs10830963	0.093	0.282	0.076	0.361	0.123	0.154	0.125	0.129	0.164	0.057	0.157	0.056
rs11257655	0.031	0.721	0.046	0.575	0.01	0.908	-0.008	0.924	0.022	0.256	0.011	0.893
rs11708067	-0.033	0.7	-0.039	0.642	-0.026	0.763	-0.029	0.731	0.033	0.7	0.037	0.652
rs11926707	-0.068	0.431	-0.065	0.43	-0.007	0.94	-0.013	0.873	-0.084	0.33	-0.09	0.276
rs1260326	-0.077	0.375	-0.072	0.395	-0.015	0.859	0.079	0.726	-0.128	0.14	-0.131	0.113
rs13266634	0.071	0.413	0.072	0.382	-0.031	0.723	-0.037	0.655	0.091	0.292	0.082	0.32
rs13342692	-0.024	0.783	-0.003	0.968	0.004	0.961	-0.024	0.771	-0.046	0.6	-0.056	0.499
rs1359790	-0.088	0.309	-0.053	0.525	0.049	0.575	-0.082	0.325	0.207	0.016	-0.201*	0.014*
rs1421085	-0.105	0.226	-0.108	0.192	0.184	0.032	0.141	0.091	0.104	0.229	0.056	0.5
rs2237895	-0.079	0.362	-0.044	0.595	0.11	0.206	0.085	0.308	0.045	0.603	0.049	0.559
rs3768321	-0.028	0.745	-0.044	0.596	0.021	0.806	0.012	0.881	0.01	0.908	-0.005	0.957
rs3802177	0.049	0.569	0.024	0.769	0.023	0.787	0.038	0.643	0.163	0.06	0.143	0.084
rs5215	0.046	0.597	0.069	0.403	0.015	0.865	-0.026	0.758	0.106	0.221	0.079	0.341
rs5219	-0.106	0.221	-0.079	0.344	0.025	0.772	-0.021	0.8	-0.036	0.683	-0.067	0.424
rs757110	0.106	0.221	0.079	0.334	0.046	0.593	0.013	0.878	0.106	0.221	0.079	0.341
rs7756992	0.038	0.658	0.046	0.586	-0.07	0.42	-0.054	0.519	-0.046	0.6	-0.025	0.767
rs7903146	-0.136	0.115	-0.146	0.075	0.279*	0.001*	0.248	0.002*	0.207*	0.016*	0.171	0.039
rs2290854	0.037	0.673	0.027	0.74	-0.037	0.666	-0.017	0.841	-0.012	0.887	0.002	0.987

SNP specific results

The following is a summary of significant findings.

rs1046314

rs1046314 (G>A) is located near *WSF1* (Flannick et al., 2019). Flannick et al., identified rs1046314 SNP to be a low risk SNP (Flannick et al., 2019). The risk allele frequency (A) was similar in those with TS to that published by GnomAd (table 7.2). Although it was not significant the dominant genotype model but did trend (p= 0.03, table 7.5). The calculated OR for rs1046314 was much lower to that reported (0.9 versus 1.1, table 7.3). Despite this, the linear regression found a positive association and T2DM status under the heterozygous GA genotype, p= 0.006. When adjusted for age and BMI the association lost significance, p= 0.058 (table 7.4). A similar pattern was observed under the dominant model. However, due to the common frequency of the risk allele it was difficult to draw any robust conclusions.

Table 7.5 the table displays the genotype frequencies in terms of percentage under the dominant genotype model for rs1046314.

	GG	AA & GA	Chi p value
NGT & no previous history controls	13.2%	86.6%	0.03
IGT/DM	28.9%	71.1%	

rs10830963

rs10830963 (C>G) is located in *MTNR1G* (Mahajan et al., 2018). The calculated allele frequencies of the TS cohort were similar to that of the reference, however, a lower frequency of risk allele G was noted in the TS controls group compared to the reference data (0.19 versus 0.29, table 7.2). Although, not significant there was a trend under the multiplicative allele model whereby those with IGT/DM had a higher frequency of the GG & CG risk genotypes (table 7.6). This was reflected in the calculated OR value which was higher than the reference (1.9 versus 1.1, p= 0.03, table 7.3). Under the non-adjusted linear regression, the dominant model

had a trending positive association (p= 0.057). When adjusted for age and BMI the p value slightly improved (p= 0.056, table 7.4).

Table 7.6 the table displays the genotype frequencies in terms of percentage under the multiplicative genotype model for rs10830963.

	CC & CG	GG & CG	Chi p value
NGT & no previous history controls	91%	19%	0.04
IGT/DM	70%	30%	

_rs1359790_

rs1359790 (C>T) is located in gene SPRY2 (Mahajan et al., 2018). The risk allele frequency was similar in those with TS to that published by GnomAd (table 7.2). There was a significant difference in the distribution of genotypes and alleles under the dominant model, p= 0.01 (table 7.7). However, unexpectedly the risk allele genotype frequencies were higher in the NGT and no previous history controls (93.5% versus 77.7%). What is more, the non-risk genotype (TT) was higher amongst those with IGT/DM (22.5%) as opposed to the NGT no history controls (6.5%, table 7.7). Although it should be noted that in each instance the non-risk TT genotype had a relatively small number of individuals especially within the NGT & no history group (n= 6). Figure 7.2 illustrates the percentage of those with IGT/DM and the three genotypes associated with rs1359790. The graphs show, that there is a higher percentage of those with TS-associated DM and risk genotypes (CT and CC), which when combined represented 77% of IGT/DM subjects versus just 20% with the non-risk genotype TT (figure 7.2).

Table 7.7 the table displays the genotype frequencies in terms of percentage under the dominant genotype model for rs1359790.

	CC & CT	TT	Chi p value
NGT & no previous history controls	93.5%	6.5%	0.01
IGT/DM	77.7%	22.2%	

Figure 7.2 Bar chart of the percentage of those with Impaired Glucose Tolerance/ Diabetes mellitus (IGT/DM) for each genotype associated with rs1359790 (C>T). The risk allele C.

Bar chart to show the percentage of with IGT/DM with associated genotypes for rs1359790
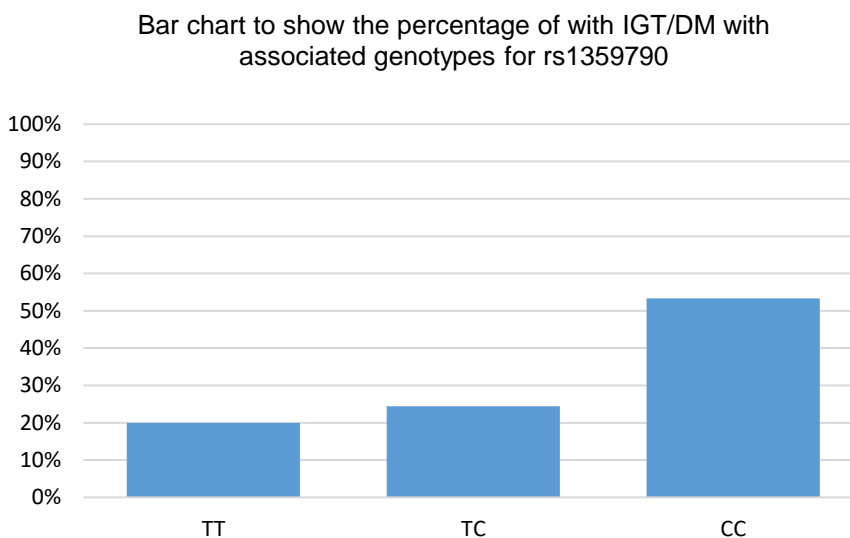
The above resulted in unusual OR and linear regression findings. The calculated OR reflected the above as it was found to be lower than that reported (0.3 versus 1.09, table 7.3). Whilst the results of the linear regression, saw a positive association under the dominant unadjusted model (p= 0.016, table 7.4), but when adjusted for age and BMI significance remained but the association was negative (p= 0.014, table 7.4).

_rs7903146_

rs7903146 (C>T) is located in gene TCF7L2, a well characterised T2DM-associated SNP (Mahajan et al., 2018). MAF as well as calculated OR were comparable to that published by GnomAd and Mahajan et al., (tables 7.2 & 7.3). Although not significant there was a trending distribution difference of risk genotypes between those with T2DM. Risk genotypes TT and TC were found to occur at a higher frequency when compared to NGT & no previous history controls (66.6% versus 46.7%, table 7.8). Table 7.8 illustrates the point as it translated into a positive association under the linear regression both the adjusted heterozygous (p= 0.002) and unadjusted dominant model (p= 0.01). Demonstrated by figure 7.3, 64.4% of those with IGT/DM has a risk genotype versus 33.3% who did not.

Table 7.8 the table displays the genotype frequencies in terms of percentage under the dominant genotype model for rs7903146.

	CC	TT & TC	Chi p value
NGT & no previous history controls	53.3%	46.7%	0.02
IGT/DM	33.3%	66.7%	

Figure 7.3 Bar chart the percentage of those with Impaired Glucose Tolerance/ Diabetes mellitus (IGT/DM) for each genotype associated with rs7903146 (C>T). The risk allele T.
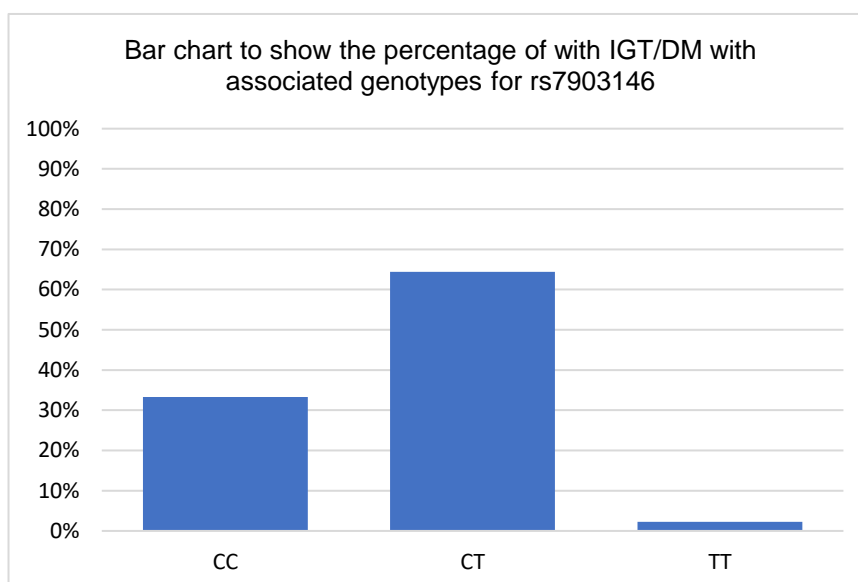
Validating & power of study 4

FTO is a well-known obesity risk gene (Babenko, Babenko, Gamieldien, & Markel, 2019).To assess the validity of the study, rs1421085 (FTO) was selected for a sub-analysis. Each additional risk allele in *FTO* is associated with a 0.39 kg/m^2 higher BMI and a 1.2-fold increased risk in obesity (Loos & Yeo, 2014). Figure 7.4 is a of rs1421085 genotypes against BMI. Although, there is significant variation in BMI in those with TS, there is a pattern of increasing BMI with the addition of each risk allele which mimics the general population.

Figure 7.4 Histogram of BMI against genotypes for rs1421085 located in well-characterised obesity associated gene-FTO. Sample size for genotype was; TT (n= 57) TC (n= 56) and (n= 23).
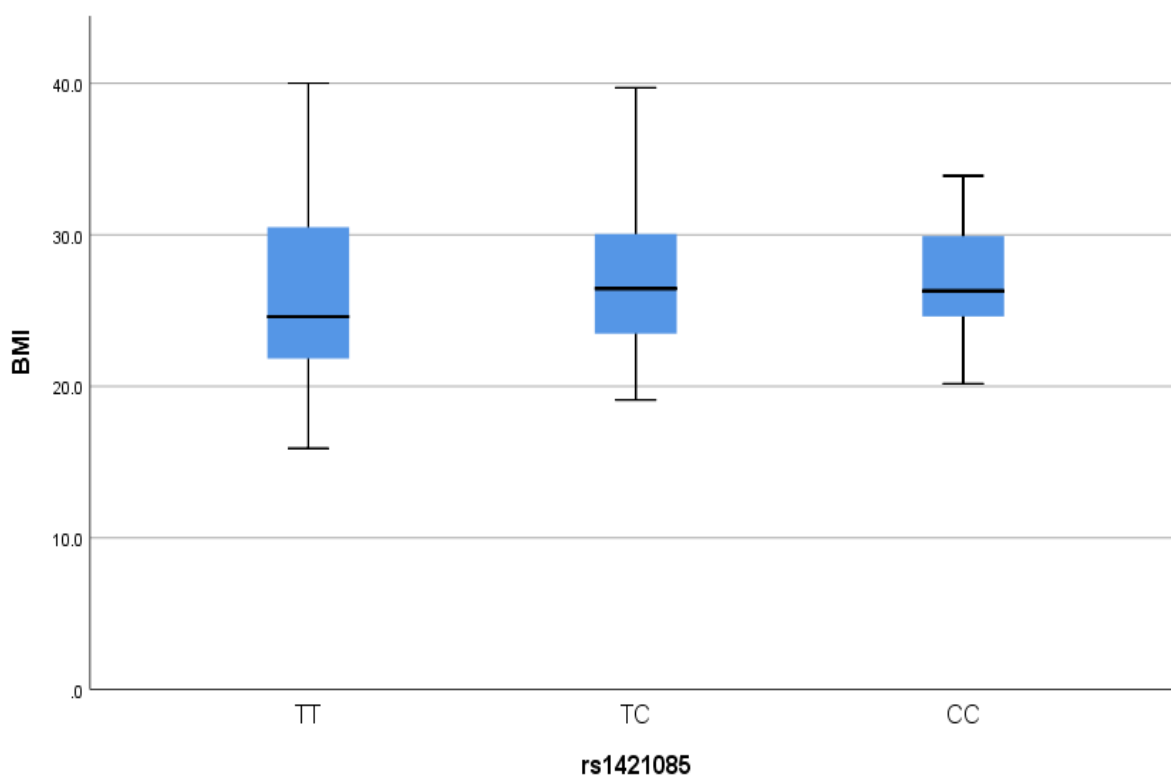
All SNPs were previously identified due to their previously identified involvement in T2DM risk and therefore had a reported OR above 1 (table 7.3). Figure 7.5 shows a forest plot of the ranked by the calculated OR with confidence intervals. A proportion of the genotyped SNPs had an OR < 1 as shown in the last 9 lines of the forest plot Figure 7.5. Thus, suggesting low power due to a small group size may have impacted some of the study findings. The forest plot displays the wide-ranging confidence intervals around the OR, which would have been narrower indicating a higher degree of confidence with a larger group size.

However, it should be reiterated that study 4 was a pilot study and previous power calculations produced by the Institute of Child Health estimated approximately 100 subjects would be needed for a valid study to detect large differences between the case-control populations. The OR of rs10830963 highlights the above and is supported by genotype data which saw a higher prevalence of those with the risk allele in those with IGT/DM (table 7.6 30% versus 19%).

Estimating future study sample size for results verification

A power calculation was performed to estimate the sample size required to verify the study findings of study 4 using the most informative SNP in this study; rs7903146. The risk allele frequency from GnomAd database is 0.28 calculated from non-Finnish European reference data (0.106 (TT) + 0.5*0.345 CT). The proportion expected to have the risk genotype is 0.38 in those with DM was calculated from the OR (OR= 1.37). Submitting this data to an online power calculator (HyLown Consulting LLC) with a power of 0.8 and error rate of 5% resulted in an estimated minimum sample size of 255 women with TS and diabetes would be needed in order to verify study findings with a ratio of 2 controls to every case.

Figure 7.5 Forest plot of the ranked calculated Odds Ratio (OR) with upper and lower confidence intervals plotted for genotyped SNPs. The red line indicates an OR of 1. SNPs are ranked from highest to lowest according to their OR.

SNP
rs10830963
rs3802177
rs5215
rs7903146
rs13266634
rs1421085
rs1046317
rs11257655
rs2290854
rs3768321
rs2237895
rs1046314
rs7756992
rs757110
rs5219
rs11708067
rs11926707
rs1260326
rs1359790
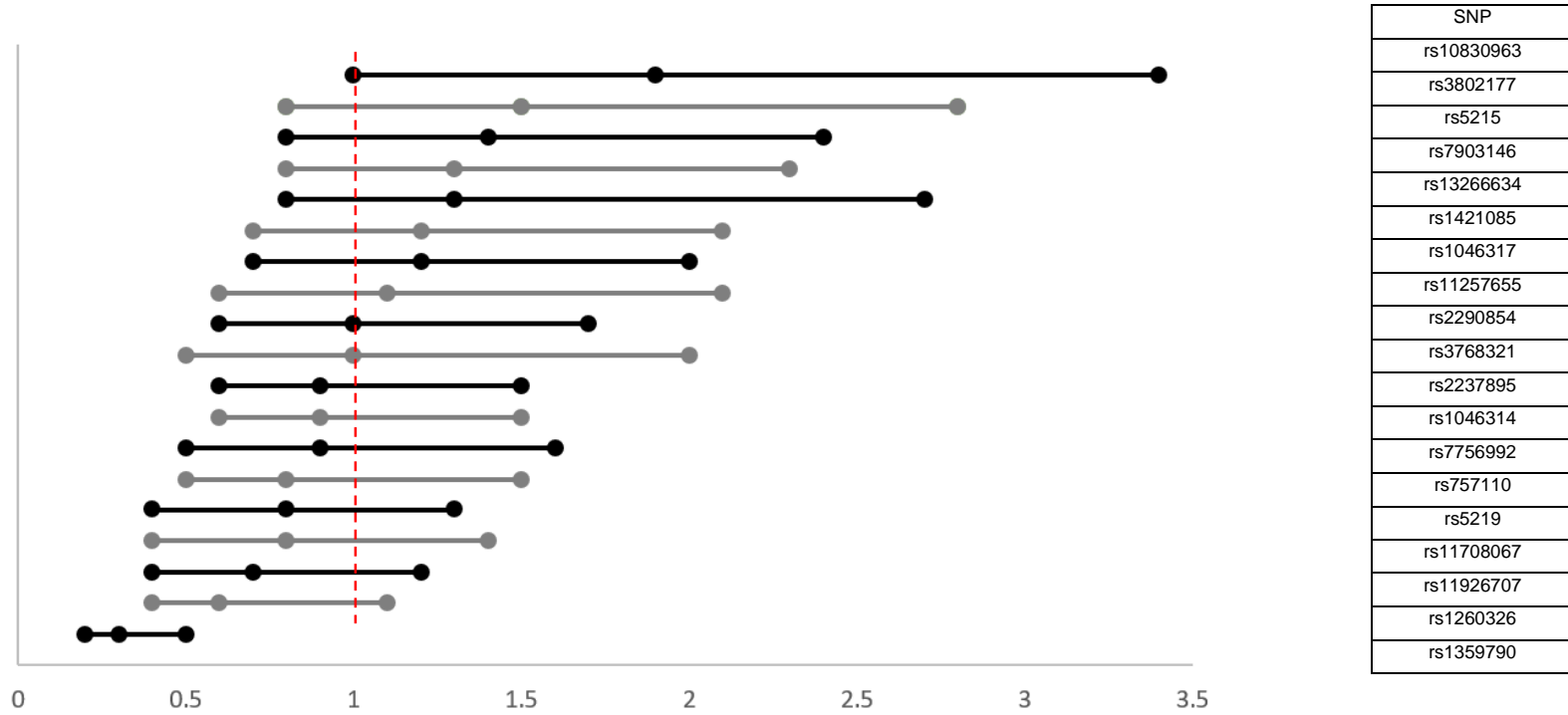
Chapter VIII

Study discussion

Turner Syndrome is a complex condition, affecting every system in the body (Elsheikh et al., 1999; Gravholt et al., 2017). In my literature review, I found a distinct lack of knowledge regarding pathogenesis of diabetes in women with TS. The focus of my doctoral work was to address the knowledge gap. In the first two chapters I undertook a characterisation of a large clinic cohort of women with TS with respect to karyotype subgroups, paediatric treatments and oestrogen use. From the karyotype analysis there were two important findings of significance.

Karyotype associations

In study 1, five karyotype subgroups were compared with respect to prevalence of various adult comorbidities. Outcomes for women with the 45,X was used as a reference to which outcomes for women from other karyotype background were compared. The karyotype distribution within the adult UCLH cohort was comparable to those previously studied (table 1.1). Except in the case of the ring and XY karyotypes, which are less frequently reported (Al Alwan, 2014; V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008; Schoemaker et al., 2008; Stochholm et al., 2006; Yesilkaya et al., 2015).

In the first instance, an analysis of paediatric outcomes was performed (table 4.2). Women with 45,X were found to have an earlier age of diagnosis and higher incidence of primary amenorrhea. Given that all other karyotype groups comprised mostly of mosaic individuals, this outcome was not unexpected and more than likely reflected the presence of the 46,XX cell line in mediating adverse outcomes. Women with 45,X/46,XX experience both better paediatric and adult outcomes demonstrating the benefit of the second cell line in mediating adverse outcomes. The exception was the equal incidence of primary amenorrhea in 45,X/46,XY individuals which mimicked that of those with 45,X. When diagnosed

with TS, those with the 45,X/46,XY are recommended for gonadectomy due to the risk of gonadoblastoma within streak gonads (Brant et al., 2006).

With regards to adult outcomes, the 45,X/46,i(X) group was found to have similar outcomes to 45,X. This result was notable because in many studies the isochromosome (Xq) has been associated with an increased incidence of hearing loss autoimmunity (Grossi et al., 2013), congenital heart disease (S. K. Prakash et al., 2016).and diabetes mellitus (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008). But observations have been variable and not found in all series.

From a genetic stand point structural karyotypes such as the isochromosome make for an interesting genetic model. The isochromosome is the result of a U-shaped strand exchange which leads to the deletion of the p arm and replication of the q arm (Harel et al., 2015). The isochromosome, like all TS-associated karyotypes, can exist along a second cell 46,XX cell line. Bakalov et al., suggested that the deletion of Xp gene constitutes as a genetic 'first hit' which then creates a susceptibility to DM (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008). Risk of pathogenesis then increases in those with the isochromosome as a 'second hit' is acquired through the presence of the triploidy of the Xq. Bakalov et al., postulated that as the X cannot undergo X inactivation, as it would in a 46,XX female leading to abnormal gene dosage (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008).

As part of study 1, I conducted a sub-analysis of 28 cases of 46,X,i(X). In those with non-mosaic isochromosome, adverse outcomes were found not to be overrepresented when compared to 45,X. In fact, those with the non-mosaic isochromosome were found to have a lower incidence of bicuspid valve and thyroid positive autoantibodies. Admittedly, markers such as hearing aid use as an assessment of hearing loss may be too insensitive and account for differences seen in this study to that of (Barrenäsa, 2000). All other outcome markers were considered robust, such as TPO positivity and thyroxine use as an indication of thyroid disease which was also found not to be increased in study 1 despite previous reports of isochromosome association (V. K. Bakalov et al., 2012; Grossi et al., 2013).

The study of Bakalov et al., was the major study showing an association between DM and iXq and I sought to clarify differences that might account for how this observation differed from study 1 where we not able to replicate the finding. First, Bakalov used data from OGTT screening in addition to recruiting established DM subjects instead of a recorded diagnosis of DM like study 1. In order to explore this further, I set out to recruit as many of the iXq cases from clinic as possible and performed OGTTs as part of screening program. Of the 28 non-mosaic isochromosome (Xq) identified 60.7% were recruited. I focussed on the non-mosaic iXq cases to investigate the effect of abnormal gene-dosage of the triploidy of isochromosome (Xq) without the mosaic variants to eliminate background noise produced by the second cell line in the isochromosome karyotypes. Despite these refinements, no overt comorbidities were associated with the non-mosaic isochromosome karyotype. By comparison, Bakalov et al., included isochromosome non-mosaic and mosaic as well as those with isodicentric chromosomes in one heterogenous karyotype group (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008). It was not stated how many of each isochromosome variant composed the overall isochromosome group. Given that in study 1 just 23% of those identified as having a non-mosaic isochromosome karyotype out of all 120 isochromosome cases, it is possible that the Bakalov et al., isochromosome variant group had a high proportion of mosaic isochromosome individuals. From the genetic point of view however, it is not obvious how the less severe karyotype groups included in Bakolov could have led to a greater phenotype.

A further issue with the 2008 study by Bakalov et al., is the means by which the cohort was ascertained. Participant recruitment was driven through advertisement thus a bias of volunteers whom potentially suspect they have diabetes or have a severer phenotype may have been created (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008). For instance, the only other comparable OGTT study conducted to date found no association between karyotype and glucose homeostasis. Ibarra-Gasparini et al., recruited women from clinic which may have led to the resulting data being more representative of the real-life expectations (Ibarra-Gasparini et al., 2018).

Another structural karyotypes associated with TS is the ring chromosome. The ring chromosome is the result of a series of breakage-fusion events creating a

ring-shaped chromosome (Guilherme et al., 2013; Hu et al., 2018). Like the isochromosome, the ring chromosome also makes for an interesting genetic model. However, due to the low frequency of the ring chromosome there is not much by way of genotype-karyotype association studies as they have often been excluded from analysis or included in heterogeneous karyotype groups (Al Alwan, 2014; V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008; Schoemaker et al., 2008; Stochholm et al., 2006; Yesilkaya et al., 2015). Van Dyke et al., was the first to report that women with the ring X chromosome were found to have a severe height and IQ deficit (Van Dyke et al., 1992). This was later confirmed by Migeon et al., who further identified that those with the smaller ring chromosome lack the *XIST* locus and therefore cannot undergo X inactivation which would affect gene dosage (Migeon, Luo, Jani, et al., 1994; Migeon, Luo, Stasiowski, et al., 1994). In contrast, Migeon et al., reported that larger ring chromosomes were associated with better outcomes due to their ability to deactivate in the presence of the *XIST* (Migeon, Luo, Stasiowski, et al., 1994). Study 1 did not examine IQ but did confirm a greater height deficit in ring chromosome subjects when compared to all other karyotype groups. Women with the ring chromosome were also found to be less likely to experience primary amenorrhea, which was expected given that all individuals within this group were mosaic.

The second important finding from study 1 was the novel association between the ring chromosome and features of metabolic syndrome. The finding raised the possibility that rX could have a gene dosage effect on diabetes risk similar to that originally proposed for iX. Metabolic syndrome was characterised by an increased incidence of elevated HbAc1 and ALT levels, with ALT likely represents fatty liver changes. There was a trend to additional metabolic syndrome associated features such as an excess of diabetes mellitus or elevated blood pressure trended in the same direction which did not achieve significance. Other cardinal features of TS such as bicuspid valve and hearing loss were found to occur at lower incidences within this group. It is important to note that the ring chromosome often occurs at a lower frequency than other karyotypes. In other smaller studies this would have meant that the ring chromosome group would have been excluded from analysis or included within a larger heterogeneous karyotype group and therefore, associations would have gone unreported. In parallel with the findings of study 1, a recent French study of 1501 girls and young

women with TS aged between 3.7 – 21.4 years found a profile of metabolic syndrome in a retrospective study of similar methodology (Fiot et al., 2019).

I set out to verify the findings of study 1 by including those with the ring chromosome in the OGTT screening. Of the 48 ring chromosome individuals identified on UCLH records, 22 were recruited (48.5%). Unlike study 1 or the data reported by Fiot et al., women with the ring chromosome were found not to have an excess of DM or an increased incidence of IGT (Fiot et al., 2019). Although genotype-karyotype retrospective studies like that of study 1 and Fiot et al., are a useful tool when researching TS, they may susceptible to false positives as there is no consistent grouping of karyotypes and with the exception of blood pressure no definitive cut-offs for outcomes. It is my belief genotype-karyotype retrospective studies should be used to guide research as it has in studies 1 and 3 but they are not sufficiently robust to be clinical useful.

The final finding from of study 1, was the that of the outcomes reported for 45,X/46,XY. As previously mentioned the 45,X/46XY group is often excluded from analysis or included within large heterogeneous karyotype groups. The 45,X/46,XY group was found to have the lowest incidence of hearing loss, hypothyroidism and a reduced aortic size index as well as a lesser height deficit. The low incidence of hypothyroidism was of particular interest as it mirrors the male-to-female ratio of this condition in the general population. I note, however, that the prevalence of autoimmune thyroid disease is still much greater than it is for men, indicating how the importance of the 45,X cell line in autoimmunity risk.

Endocrine treatments and comorbidities in women with Turner Syndrome

There are two major hormones which girls and women with TS are prescribed; GH as a child to help achieve optimal adult height then oestrogen to initiate puberty and from its initiation onwards to prevent oestrogen deficiency (Gravholt et al., 2017). The effects of which were examined against adult outcomes as part of study 2.

GH is prescribed in paediatric years for the optimisation of final adult height and is reported to have continued metabolic benefits after treatment discontinuation (Bannink, van der Palen, Mulder, & de Muinck Keizer-Schrama, 2009; Gravholt et al., 2017). Bannink et al., examined metabolic parameters in young women who had an average treatment duration of 8.7 years. In the small study of 39, GH therapy was reported to have beneficial effects on lipid serum even after discontinuation. GH induced insulin insensitivity which seem to continue after GH therapy. However, authors postulated that this may be the result of having TS as opposed to GH therapy (Bannink et al., 2009). A later 7-year follow up study of 104 patients found no such effect (Baronio et al., 2017). Study 2, examined the use of GH against adult health outcomes. Approximately 56.6% of the women in study 2 were found to have used GH in paediatric years and an age effect was identified, by which women under the age of 35 years received GH in childhood more frequently than those over the age of 40. Although study 2 differed in methodology by using cross-sectional data, the data was found to confirm no association between GH use or treatment duration and metabolic parameters.

In recent years, there has been growing body of evidence to support an earlier age of oestrogen therapy initiation in regard to improved bone outcomes in adult life (Nguyen et al., 2018; Nguyen et al., 2017). In the era of major studies on the effectiveness of GH in girls with TS, induction of puberty was often delayed for fear of reducing final height with early closer of the long bone epiphyses. In recent years, there has been growing body of evidence to support an earlier age of oestrogen therapy initiation with regard to improved bone outcomes in adult life and also to the timing of sexual debut and developing relationships (Carel et al., 2006; Nguyen et al., 2018; Nguyen et al., 2017). In study 2, I explored the relationship between the timing of introduction of oestrogen with bone density, the main oestrogen dependent health outcome in routine clinic data. Data

produced as part of study 2 supported this showing a higher t-score in those whom started oestrogen earlier (figure 5.1). BMD data from study 2 was used alongside historical bone fracture data obtained from the life course project questionnaires to compare bone outcomes for women with TS and POI (Cardona Attard et al., 2019). Women with TS were found not to experience an excess of fractures when compared to oestrogen deficient women with POI. However, women with TS were found to experience more from major fractures such as those of the vertebrae which is believed to related to hearing impairment, thin cortical bone and abnormal bone remodelling (Cardona Attard et al., 2019). Combining the data on bone density and fracture, I conclude that earlier induction of puberty is likely to benefit bone strength in women with oestrogen deficiency but that larger studies of fracture risk are required.

Current guidelines suggest ORT should be taken until the age of 50 and then slowly withdrawn to mimic the natural history of oestrogen within the general population (Gravholt et al., 2017). ORT use data presented as part of study 2 from the TLCP gave a novel insight into ORT use throughout the life course of girls and women with TS (figure 5.2). Women tended to transition from oral ORT to TE with advancing age and more than likely an increasing accumulation of comorbidities such as hypertension and obesity (figure 5.2). The OCP was associated with adverse outcomes including raised blood pressure, LDL cholesterol and triglycerides as they have been shown in the general population (Chasan-Taber et al., 1996; Josse, Garcia-Bailo, Fischer, & El-Sohemy, 2012).This would suggest that OCP use in TS should be limited to contraception or control of menstruation but not used as a routine form of oestrogen replacement.

Metabolic comorbidities in women with Turner Syndrome

Non-Alcoholic Fatty Liver Disease (NAFLD) is a common finding in women with TS, initially presenting as elevated liver enzymes (Koulouri, Ostberg, & Conway, 2008). Elevated liver enzymes were associated with the ring chromosome as part of the metabolic syndrome phenotype. Following on from this as part of study 2, differing ORT administration modalities were investigated. TE users in study 2, were shown to have an elevation in liver enzymes when compared to other user subgroups which persisted after controlling for obvious confounders such as BMI. It is hypothesised that this elevation in liver enzymes reflects the oestrogen-sensitive nature of the liver, which is bypassed with TE use (Gravholt, Poulsen, Ott, Christiansen, & Vilstrup, 2007). Therefore, there is a balance to be made between the benefits of TE use in terms of lower risk of thrombosis against a possible risk of progressive liver disease. The natural history of is yet unclear. Architectural changes to the liver such as cirrhosis with a risk of 5.6% in TS, is reported to increase with age (Idilman, De Maria, Colantoni, Kugelmas, & Van Thiel, 2000).and a histological-based report found cirrhosis to be more frequent in those individuals with TS and prolonged oestrogen deficiency (Roulot et al., 2004).

The metabolic data and ORT data from study 2, when combined, demonstrated how there may be a trade-off to be considered when prescribing OE versus TE in TS. That is managing the risk of thrombosis which increases with the onset of hypertension and obesity when taking OE and that of NAFLD with an unknown trajectory when considering TE.

Characterisation of diabetes mellitus in women with Turner Syndrome

Following on from studies 1 and 2, I chose to focus on the characteristics of diabetes in women with TS because this was an important modifiable risk to health and it was not clear that the classical characterisation of diabetes applied to this cohort. As many of the women with established diabetes as possible were recruited as part of study 3. When studying comorbidities in TS it is important to account for oestrogen deficiency therefore an oestrogen deficient reference range was established by recruiting women with POI. Girls and women with POI experience oestrogen deficiency from unknown cause which largely been found

not to be genetic. Unlike women with TS, comorbidities in POI seem to relate to the effects of oestrogen deficiency when compared to TS.

Table 8.1 summarises the salient findings from study 3 compared to two similar studies on glucose metabolism in women with TS. The main focus was to explore similarities between DM in women with TS compared to the other commonly used categories. T1DM is characterised as an early onset, insulin dependent autoimmune condition whilst T2DM represents a late onset, non-insulin dependent disease with lifestyle and genetic factors compounding risk. LADA shares features of both T1DM and T2DM. LADA, like T1DM has an autoimmune element and those affected will eventually require insulin. Unlike, those with T1DM, those with LADA will experience an adult onset and disease progression may be accelerated by T2DM risk factors such as obesity.

Girls and women with TS have been reported to have an 11-fold increased risk for type 1 and 4-fold increased risk for type 2 diabetes when compared to the general population (Gravholt, Juul, et al., 1998). The calculated risk of DM in TS is derived from a 1998 Danish registry study of 594 women with TS. Issues regarding diagnosis and registry studies were raised by Bolar et al., as no information is given regarding the criteria used to make the diagnoses (Bolar, Hoffman, Maneatis, & Lippe, 2008). Other studies since have not reported such high incidences of T1DM (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008; V. K. Bakalov et al., 2004; Sun et al., 2019). Just one subject reported to have adult onset T1DM in the ULCH diabetic population. Interestingly, this was identical to that reported by (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008).

The occult IGT and DM risk of DM in the ULCH cohort was respectively 19.5% and 7.8%. The lower incidence of IGT/DM can be account for by the marginally smaller number of OGTT conducted in the prospective phase of study 3 when compared to the previous studies highlighted in table 8.1 and the means of cohort ascertainment (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008; Ibarra-Gasparini et al., 2018). Focussing on the former, Ibarra-Gasparini conducted a 12-month follow-up OGTT screening programme of 113 women with TS. Women were recruited from clinic and screened twice over a period of 12 - 24.5 months. At the first assessment a number of women were found to have IGT

(7/113) or DM (5/113). Those with DM were excluded from the second screening. After the second screening, 3 of the 7 cases (42.9%) of IGT went on to develop DM with an overall rate of newly diagnosed DM of 12.4% (Ibarra-Gasparini et al., 2018). Study 3 did not have a follow-up screening. But it is my assumption based on the Ibarra-Gasparini data, that if screened again 6 out of the 15 women from study 3 with IGT would be found to be diabetic at a subsequent OGTT.

Table 8.1 shows the characteristics of OGTT screening programme conducted as part of study 3 against that of other major OGTT studies.

	Ascertainment	Total N	Cohort Mean Age	Prevalence (%)	
				IGT	DM
Study 3	Clinic	106	35 (18.2 - 70.1)	19.5	7.8
Bakalov et al.,	Advertised	224	35.4 (18 - 67)	23.4	13.4
Ibarra-Gasparini et al.,	Clinic	113	32 (20 - 61)	23	12.4

Average age of DM onset in study 3 was 36.1 years (range 11- 56 years). This age was comparable to that published by (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008; Ibarra-Gasparini et al., 2018), which placed age of DM onset between 38 and 40.5 years. Figure 6.1 showed the cumulative frequency of the age of onset for all DM subjects studied as part of study 3. The frequency of T1DM and T2DM were also plotted as a means of comparison. The age of TS-associated DM was found to be between T1DM and T2DM, with 50% of those DM subjects in study 3 being diagnosed before the age by 32. This pattern of age of onset of DM is very slimier to that found in Latent Autoimmune Diabetes in Adult (LADA). The Immunology of Diabetes Society defines LADA as 1) adult onset ≥30 years 2) presence of any islet cell autoantibody and 3) absence of insulin treatment for the first 6 months after diagnosis(Leslie, Williams, & Pozzilli, 2006).With age of onset in mind, TS-associated DM is certainly more reminiscent of LADA as opposed to other adult onset DM types like T2DM.

A positive family history of DM is a common feature of T2DM that is independent to lifestyle, anthropometric and individual genetic risk factors (InterAct et al., 2013). The same is also true of LADA (Carlsson, Midthjell, & Grill, 2007). Despite

many of the features of T2DM including increased waist circumference and a higher BMI woman with IGT/DM were found not to have a higher prevalence of first degree affected relatives.

Ethnicity is also considered as a risk factor for T2DM. Among ethnic communities in the U.K. the prevalence of T2DM is 3 – 5 times higher than that of the white British population and significant proportion of those diagnosed are so before the age of 40 (Goff, 2019). Women in study 3 self-reported ethnicity at the time of testing and 4 major group were identified; white European, African descent, South Asian and other which represented group of women who identified as bi-racial or otherwise. Around 74.7% of the women in the TS cohort reported to be of European descent. Other ethnic groups represented 25.8%. This was higher than that reported in the most recent census in the UK (Goff, 2019). There was no difference in the distribution of IGT/DM between across ethnic groups in study 3. However, given a small number of women represented non-European ethnic groups in this cohort, it is difficult to be conclusive on the influence of ethnicity in TS-associated DM.

Insulin resistance has been reported to be an intrinsic defect in TS (Gravholt, Naeraa, et al., 1998; Salgin et al., 2006). Data from study 3 supported this finding; women who were NGT were found to have normal fasting insulin as well as HOMA-IR. whilst women with IGT/DM were found to have higher fasting insulin as well as higher HOMA-IR representing change towards IR. Ibarra-Gasparini reported a similar pattern of increasing IR when transitioning from NGT to IGT/DM (Ibarra-Gasparini et al., 2018). However, this finding did not translate when comparing NGT to POI controls, who were found to have a similar degree of IR experienced those with IGT/DM TS subjects. The inconsistency could be the result of a sampling error as few women POI controls were recruited (n= 15). Women with POI attend clinic less frequently than those with TS as they experience less comorbidities in comparison. Therefore, women with POI who do attend clinic may represent those with a severer phenotype and will not be representative of the POI populations as a whole. A number of individuals within the POI group were noted to have increased fasting insulin which, more than likely, resulted in a data skew for the fasting insulin dependent HOMA-IR calculation in the small POI group. C-peptide was normal to be within a normal range for the POI group when compared to NGT TS women thus supporting the

data skew hypothesis. Just one other TS OGTT study to date has included POI controls (V. K. Bakalov et al., 2004) and whilst HOMA-IR was not calculated all other parameters investigated regarding insulin were considered within the normal range. Further research would be needed in order to detangle this finding.

C-peptide and DM-associated autoantibodies such as GAD, IA-2 and ZnT8 can be utilised to differentiate DM subtypes (Leslie et al., 2016). C-peptide assesses beta-cell function and therefore insulin secretion; low c-peptide levels would indicate T1DM whilst, those with T2DM would be expected to have high c-peptide levels representing IR (Jones & Hattersley, 2013). Women with TS were found to have normal c-peptide levels when glucose tolerance was normal compared to women with POI. Higher c-peptide levels were found in those diagnosed as IGT and newly diagnosed DM indicating changes towards IR rather than reduced insulin secretion when compared to oestrogen deficient controls. The c-peptide profile noted in women with TS was more representative of those with T2DM as opposed to T1DM.

In autoimmune DM subtypes, beta-cell function is modulated by GAD autoimmunity; those who are GAD positive will see a quicker progression towards insulin dependency as a result of a more rapid decline in beta-cell function (Hagopian et al., 1993). GAD titres are highest among those with T1DM and lowest in those with T2DM (Hawa et al., 2009; Leslie et al., 2016). Previous studies have linked TS to an excess of autoimmunity (Gawlik et al., 2018; Grossi et al., 2013; Jorgensen et al., 2010; Mortensen et al., 2009), which was confirmed in by study 3 as women with TS were 8.5 times more likely to be GAD positive than the general population (McDonald et al., 2011). When observing the diabetics alone 22% were GAD positive. Which was higher than that reported by Bakalov (2008) who also measure GAD autoantibodies DM subjects, 3/57 (5.3%) (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008; Ibarra-Gasparini et al., 2018; Mortensen et al., 2009). Whilst the frequency of GAD positivity may not be as high the estimated 68.6% of those with LADA (Pozzilli & Pieralice, 2018). it is still considerably higher than that of T2DM (Hawa et al., 2014). Positivity for other DM-associated autoantibodies tested, ZnT8 and IA-2, were rarely positive in TS and no found at all in those with POI (Howson et al., 2011).

GAD positive autoantibodies are associated with an earlier age of DM onset (Howson et al., 2011). Age of onset in those with TS-associated DM did not differ between GAD positive and negative subjects. However, in study 3 only 6 subjects were GAD positive with diabetes and this was too few to be able to draw a conclusion about age of onset trends. In those with established DM, GAD positivity was associated with the requirement of insulin therapy and therefore low beta-cell function even though just one subject was reported having T1DM. Interestingly, three individuals were found to be NGT normal and GAD positive and it will be interesting to follow these cases in the future to gain an idea of the predictive power of GAD autoimmunity. A similar finding was reported by Bakalov et al,. who found 5/113 women tested were NGT normal and GAD positive. Finally, longitudinal studies have demonstrated how GAD positivity is transient (Rasouli et al., 2013; Robert Turner et al., 1997). Therefore, it is entirely plausible for GAD positivity to be higher in the TS population than previously reported.

Typically, increased waist circumference and a higher BMI are associated with T2DM (Feller et al., 2010). As expected women with IGT/DM were found to have an increased waist circumference and higher BMI when compared to all other groups. However, lifestyle risk factors are not limited to T2DM. Most recently, a Swedish study found those with LADA and lower GAD titres had a higher degree of IR associated with an increased BMI (Hjort et al., 2018). Which suggests there are other mechanisms other than autoimmunity behind LADA pathogenesis (Leslie et al., 2016). Current TS guidelines recommend annual fasting blood glucose and HbAc1 tests (Gravholt et al., 2017). OGTT are to be considered when HbAc1 is elevated. Ibarra-Gasparini et al., reported there was no correlation between fasting glucose and glucose after 120 minutes or HbAc1 in a large-scale TS OGTT study, which was confirmed by study 3 (Ibarra-Gasparini et al., 2018). I further found that that BMI has greater potential for identifying those at risk of TS-associated DM.

Researchers today often consider DM subtypes to represent a continuous spectrum between two very different disorders of glucose homeostasis T1DM and T2DM (Brooks-Worrell & Palmer, 2011; Leslie et al., 2016). As demonstrated in table 8.2 TS-associated DM shares features and risk factors of both LADA and T2DM. Therefore, I conclude that TS-associated DM lies between somewhere on the DM spectrum between LADA and T2DM with both GAD autoimmunity and

T2DM risk factors such as obesity and IR playing a role in pathogenesis. International guidelines do not presently recommend for routine OGTT or GAD autoantibody testing. As a result of this study, I would consider women with TS and a BMI ≥ 27 to be at a higher risk of TS-associated DM and should be prioritised for OGTT in the first instance. The 5-yearly implementation of GAD screening alongside current autoantibody testing, could further stratify those in need of an OGTT or in those whom are recognised as IGT or DM identify those who may need insulin in the future and may require closer monitoring (Hagopian et al., 1993). Further future work is needed to identify how many IGT women with TS go on to develop DM in order to refine how frequent OGTT should occur.

Table 8.2 shows the features of 3 characterised subtypes of Diabetes mellitus (DM) against that of Turner Syndrome-associated DM. Reference data was extracted from a variety of sources (Brahmkshatriya, Mehta, Saboo, & Goyal, 2012; Hawa et al., 2009; Hjort et al., 2018; Leslie et al., 2016).

Features	T1DM	T2DM	LADA	TS-associated DM
Age of Onset ± SD	12.55 ± 1.34	48.01 ± 0.5	33.4 ± 2.15	35.8 ± 10.9
Prevalence of GAD	85%	7%	68.6%	22.2%
Insulin Resistance	32%	89%	42%	51.7%
Insulin therapy	Always	Sometimes	Eventually	17.2%
C-peptide	Low	High	Variable	Variable
Inheritance pattern	Sometimes	Yes	Yes	Unknown
Obesity risks	No	Yes	Yes	Yes
Associated with other AD	Yes	No	Yes	Yes

In order to explore the relative importance of BMI and insulin resistance I performed regression analysis of the data in study 3 comparing women with normal glucose tolerance with those with IGT or DM and controlling for age. BMI and HOMA-IR found that each were associated with IGT/DM in TS. Furthermore, each of these factors were found to be independent with BMI and HOMA-IR equally contributing towards the variance. I conclude from this analysis that a

factor causing insulin resistance independent of obesity is an important contributor to diabetes risk and consider use of oestrogen to be one such factor.

Oestrogen is now recognised as an important modulator of glucose homeostasis, insulin secretion and energy balance (Mauvais-Jarvis, Clegg, & Hevener, 2013). There few studies that have explored the effect of oestrogen on glucose homeostasis. Studies conducted in menopausal women have found an association between T2DM and an earlier age of menopause (Brand et al., 2013; S. K. Park et al., 2017). Furthermore, previous studies have demonstrated an increased in insulin resistance (Poehlman, Toth, & Gardner, 1995; Razay, Heaton, & Bolton, 1992).and fasting glucose (Dallongeville, 1995; Lynch, Ryan, Berman, Sorkin, & Nicklas, 2002) in menopausal women when compared to perimenopausal counterparts. IR in the menopausal population has been linked to increased BMI and visceral fat accumulation combined with a reduced insulin sensitivity with age (Carr, 2003).

The majority of women with TS will require ORT. As part of study 2, TE users were found to have elevated HbAc1, which at the time of my publication was believed to reflect prescription bias as TE will have been the primary ORT prescribed for overweight individuals. However, whilst HbAc1 is not a direct measure of insulin, it is interesting that TE use within the menopausal population has been associated with insulin resistance when compared to subjects taking oral oestrogens (de Lauzon-Guillain et al., 2009; Mauvais-Jarvis et al., 2013). As previously mentioned in relation to NAFLD, reduced IR in oral oestrogen users more than likely reflects the action of oestrogen on the liver which is bypassed when using TE. A possible explanation that draws together these observations on the effects of oestrogen deficiency and its replacement with the results of studies 2 and 3 is that women with TS may be relatively under-oestrogenised or resistant to the oestrogen contributing to insulin resistance. Following this thought through, higher doses of oestrogen replacement might reduce the risk of diabetes in women with TS. Evidence supporting this theory, can be found in studies of pubertal development. Girls with TS were found to have a poor response to estradiol in terms of the size of the uterus when compared to other groups with hypogonadism such as POI, despite the utilisation of a standardised protocol (Burt et al., 2019).

Common T2DM risk SNP variants in women with TS

TS is a potentially important model to explore the genetic pathway leading to risk of acquiring DM. As study 3, did not identify a strong autoimmune association with DM in women with TS there remains the possibility that lack of X chromosome material is responsible for excess diabetes risk. Previous work by Prakash et al., and Corbitt et al., has demonstrated how the missing X chromosome genes may interact with common autosomal variants to increased risk of congenital cardiovascular anomalies (Corbitt et al., 2018; S. K. Prakash et al., 2016). T2DM is a well-characterised condition with abundance of genomic research, with many risk SNP identified by on the autosomes by GWAS (Alonso, 2019). The literature review highlighted a number of X-linked genes and SNPs involved in DM; *FOXP3,* D12S853, rs146662075, *AGTR* and *INSR4* (Bonas-Guarch et al., 2018; Kadowaki, 2000; M. Liu et al., 2015; C. Shao et al., 2013).

To explore the possibility of interactions between the X chromosome and common T2DM associated autosomal variants, a pilot case control study was conducted in women from study 3. DNA samples were taken from those who took part in study 3 at the time of OGTT and established DM. Additional, samples were obtained from the adult TS clinic from women with no previous history of IGT or elevated HbAc1.GWAS has implicated 40 loci in T2DM pathogenesis (Alonso, 2019). This metanalysis conducted within the period of 2018 - 2019 used the *Nature* search engine. Just two publications met the search criteria. From this reference I drew up a list of 19 SNPs. SNPs were selected based being a previously reported T2DM-associated SNP with a Minor Allele Frequency above ≥20% and its presence on the Infinium Global Screening Array v3.0.

Of the 19 SNP investigated only one stood out to be influential for diabetes risk.. The most notable finding relating study 4 is that the SNP rs7903146 was associated with IGT/DM when compared to women without DM. The OR value for rs7903146 was 1.3 and ranked second highest of the genotyped SNPs (figure 7.5). Variant rs7903146, is located in *TCF7L2* and has been widely reported for its strong association with T2DM-risk (Grant et al., 2006; Voight et al., 2010). Those carrying variation within *TCF7L2* are 50% more likely to develop T2DM (Cauchi & Froguel, 2008). Encoding for a transcription factor, *TCF7L2* features heavily in the Wnt/β-catenin signalling pathway which plays a vital role in; cell

proliferation, differentiation and apoptosis, as well tissue homeostasis and metabolic processes (Smith, 2007). As expected from its association with T2DM, TCF*7L2* is highly expressed in many glucose sensing and metabolising tissues (Boj et al., 2012; Cauchi et al., 2006; W. Shao et al., 2013). The exact mechanism which by non-coding rs7903146 increases T2DM risk is still not understood, but it is known to effect beta-cell function and islet cell morphology (Chen et al., 2018; Le Bacquer et al., 2012; Renstrom, 2012; Shu et al., 2008).

Genomic work supports the theory that DM is a continuous spectrum between T1DM and T2DM (Cervin et al., 2008). To date, several distinct susceptibility loci for both T1DM and T2DM have been identified (Bradfield et al., 2011; Replication et al., 2014; Wellcome Trust Case Control, 2007). In terms of the DM spectrum, LADA has an intermediate genetic architecture of both T1DM and T2DM (Mishra et al., 2017). Those with LADA have been reported to carry both protective and susceptibility alleles associated with T1DM in *HLA*, coded for by the Major Histocompatibility Complex (MHC), as well as T2DM susceptibility genes like *TCF7L2* that I identified in women with TS (Andersen et al., 2014; Hosszufalusi et al., 2003; Tuomi et al., 1999). Studies comparing LADA subjects to those with adult onset T1DM, found those with LADA carried more T1DM protective alleles than those with adult onset T1DM and vice versa (Hosszufalusi et al., 2003). Thus suggesting, that early onset T1DM could be the result of having more T1DM susceptibility genes as opposed to those conferring with protection (Hosszufalusi et al., 2003). T1DM genes were not investigated as part of study 4 so it is difficult to anticipate their role in TS-associated DM. However, it is interesting that in T1DM subjects those with rs7903146 have been found to have beta-cell persistence (McKeigue et al., 2019), which may explain why women with TS-associated DM profile of having an autoimmune-like DM with no obvious decline in beta-cell function. Furthermore, obesity genes such as *FTO* have been demonstrated to have combination effects with *TCF7L2* and HLA in the promotion of LADA.

The other variant of biological interest was rs10830963 located near *MTNR1B*. Variants near *MTNR1B* have been shown to negatively influence fasting glucose in European populations (Bouatia-Naji et al., 2009). The *MTNR1B gene* has been shown to be expressed in pancreatic cells and may be involved in the mediation of the effects of melatonin on basal and glucose induced-insulin release

(Ramracheya et al., 2008). In studies of LADA, rs10830963 has been shown to be strongly associated with low GAD titres and is more likely to be present in those with T2DM as opposed to LADA subjects (Andersen et al., 2014). GAD titres in relation to BMI were not examined due to the relatively small number of GAD positive subjects (n= 9). The allele frequency for rs10830963 was comparable between the groups, however under the multiplicative model there was a lower proportion of individuals for the risk allele G (30% versus 19% table 7.6). The OR of rs10830963 was higher than that previously reported and ranked highest amongst the TS population, (OR 1.9 versus 1.1 versus 7.3, table 7.3 & figure 7.5). A linear regression analysis of this SNP showed a trend towards diabetes risk under the dominant model that did not achieve significance (p=0.056, table 7.4).

FTO is a well-known obesity-associated gene identified early on in the days of GWAS (Dina et al., 2007; Loos & Yeo, 2014) and due to the relationship between obesity and T2DM, FTO has since been linked to T2DM (Mahajan et al., 2018). I used rs1421085 to assess the validity of study 4 due to the known effect of the risk genotypes on BMI (Chang et al., 2008). Figure 7.4 demonstrated the established stepwise increase in BMI with the addition of each risk allele thus confirming the methodology behind study 4 was valid which did not achieve significance.

With the genetic architecture of LADA in mind, I propose that TS-associated DM is caused by a deficient X chromosome genes or rare variation and is modulated by the presence of T2DM genetic variants accounting for T2DM associated risk factors I observed within the cohort. The two hit hypothesis is not new as it was first described by Bakalov et al., but this is the first piece of evidence to support this theory in TS-associated DM (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008).

Study 4 was intended to be a pilot study of this methodology and as such it was apparent that low power manifested in several ways. Inconsistencies were found between reported and calculated OR for almost all SNPS (table 7.3). Calling attention to the issue of low power were the results of rs1359790 as they are significant but in the reverse risk to that expected. Displayed in table 7.7 are the results for rs1359790, women with IGT/DM were reported to have a higher

incidence of the non-risk allele T (figure 7.2) and an adjusted linear regression saw a negative association between the risk allele and T2DM status. A similar conflicting pattern of results was also noted for rs1046314 whereby risk allele genotype prevalence was higher in the non-affected, a lower than previously reported and a positive association with T2DM under the heterozygous model.

A power calculation conducted by the Institute of Child Health (figure 7.1) estimated that in order to achieve a power of 0.8 would require a cohort of at least 100 with a ratio of 2 controls to every IGT/DM to detect large significant differences in allele frequencies between cases and controls. The calculation was based on the previously established rate of IGT/DM of around 20% (V. K. Bakalov, Cheng, C., Zhou, J., and Bondy, C.A., 2008; Ibarra-Gasparini et al., 2018) and if the SNPs investigated were deemed to be common within the general population so to improve power (MAF ≥20%). As part of study 4, 137 individuals were genotyped and some associations were made thus proving the calculation to hold true to in practice. However, a second power calculation was performed based on the allele frequency of risk genotypes in the IGT/DM versus that of the NGT subjects for rs7903146 using the same power and case control rations estimated approximately 255 subjects.

Achieving a TS cohort of such size would be fraught with issues yet not impossible as in study 3 I found a disconnect between fasting glucose and glucose after a 120-minute OGTT (table 6.1). In the majority of T2DM studies in the general population, subjects with no history of IGT or DM with HbAc1 and fasting glucose within the normal range are considered to be NGT. Therefore, future studies would require each TS subject to be screened via OGTT in order to rule out IGT/DM and as over ¼ of women tested via OGTT had IGT/DM it would mean a larger number of women than anticipated would been to be recruited. When combined the results of the more recent power calculation using the TS genotype data and study 3 suggest the importance of conducting such pilot studies.

GWAS has been successful in identifying many common variants associated with DM (Alonso, 2019). So far GWAS findings have not translated into major clinical advances due to the relatively due to the relatively weak effects of the identified variants (Alonso, 2019). Ever-growing multi-ancestry cohorts, re-analysis of

current data and meta-analysis seem to be one of the few available means of combating the above. For example, a recent re-analysis of existing GWAS data combined with genomic imputation lead to the identification of a rare Xp23 variant rs146662075, which was known to be involved in the modulation of insulin sensitivity and is associated with a 2-fold increased risk of T2DM in males (Bonas-Guarch et al., 2018). This was the first study of its kind to provide extensive coverage of the X chromosome (Bonas-Guarch et al., 2018) which has otherwise been absent in previous GWAS studies (Wise et al., 2013).

The X chromosome has been proposed to be the source of missing heritability for many complex diseases (Anguita-Ruiz et al., 2019). Presently, it is hard to estimate X chromosome involvement in any complex disease due a number of issues discussed in the literature review (Wise et al., 2013). Exome sequencing may provide researchers with an exciting new opportunity to explore the X chromosome (Mondal, Shetty, Patel, Cutler, & Zwick, 2011) and complex disease (Kiezun et al., 2012). Examining exomes allows for the identification of rarer more causative variants (Kiezun et al., 2012) and as previously mentioned has already been proven effective in identifying X-chromosome and autosomes interactions (Corbitt et al., 2018).and T2DM (Flannick et al., 2019).

To date, very few studies have used genomic technologies in TS comorbidity research (Corbitt et al., 2018; S. K. Prakash et al., 2016). Study 4 was the first study to examine the genetics of TS-associated DM. I believe it demonstrates, along with previous studies, that genomic technologies need to be included more often in TS research if we are to advance our understanding of the molecular phenotype of TS. The implications of understanding, the molecular phenotype of TS is not limited to TS but also for the wider population.

Conclusions & Future Research

Overall, I believe studies 1 - 3 have shown TS-associated DM to be a singular entity on the DM spectrum residing between LADA and T2DM. Like those with LADA, women with TS experience an early onset of DM which may be exacerbated by lifestyle risk factors such as obesity. But beta-cell decline and autoimmunity does not seem to role in the aetiology in TS-associated DM as it does in LADA. Further research is now needed to further clarify the DM phenotype. A larger follow-up study, like that conducted by Ibarra-Gasparini et al., will be vital in identifying how many with IGT will go on to develop DM and the role of GAD autoantibodies in identifying those at-risk or predicting insulin requirements in the future. Moreover, data from an expanded OGTT screening programme in gaining a representative view of IGT and DM prevalence in TS. A study such as this would need to be multicentre. The U.K. would provide an ideal setting for a collaborative multicentre approach as there are several adult TS clinics serving the majority of the country. Not to mention, many of the adult TS clinics in the UK have close ties with the Turner Syndrome Support Society which could prove advantageous when recruiting women whom do not attend clinic regularly or are under the clinical management of general endocrine services. Contacting women under other endocrine services will prove important for identifying the currently under represented T1DM in order to establish the true risk of T1DM in TS.

The question still remains about the role of oestrogen in IR. Combined oestrogen and OGTT studies are rare with many studies comparing ORT modalities on glucose and insulin homeostasis (Alves et al., 2006; Gravholt, Naeraa, et al., 1998; Torres-Santiago et al., 2013). Burt et al., in a study of girls with TS found that, even with a standardised induction of puberty protocol, girls with TS did not respond in the same manner as other girls with hypogonadism. The authors concluded that girls with TS are under-oestrogenised and may require a higher dose of oestrogen to achieve optimal results such as uterine development (Burt et al., 2019).

A study investigating the role of oestrogen in glucose and insulin homeostasis would be complex. However, a follow-up study of girls with TS from pre-induction of puberty to adulthood implementing routine OGTT and serum estradiol

screening at each interval would enable researchers to see the effect of oestrogen as a whole. Furthermore, exposing subgroups of girls to different doses of oestrogen will add further detail as to whether or not the concentration of oestrogen plays a role. In light of the unexpected findings of a higher degree of insulin resistance of those with POI, I would propose that girls with POI should be included in such a study in order to verify the true relationship between oestrogen and IR.

The future of research in TS should be driven by genomic technologies with retrospective studies guiding research themes. As demonstrated by study 4, GWAS studies are possible in TS but issues surrounding power which exist even in large scale studies and will only be exacerbated in smaller TS cohorts.

On the other hand, exome sequencing (ES) could provide an exciting new era in TS research. ES allows for the identification of rare and common variants at a single nucleotide level (Li, Samuels, Zhao, Shyr, & Guo, 2018) as well as copy number variation (Posey, 2019). Corbitt et al., has proven that ES studies are not only feasible but effective do not require large cohorts. In 2019, Corbitt conducted the first ES comorbidity study in TS in just 188 subjects and yet yielded intriguing results discovering association between common autosomal variants in *TIMP3* found in the general population and X-chromosome *TIMP1*. There is an issue regarding the sensitivity of ES to detect triploidy (Posey, 2019). Researchers overcame the issue of triploidy by recreating molecular karyotypes to create an average copy number, for instance if 50% of cells were 45,X and the other 50% were found to be 45,X/46,XX the average copy number would be 1.5, which would be expected to result in a higher average plasma protein level (Corbitt, Gutierrez, Silberbach, & Maslen, 2019). Although karyotype frequencies were not reported so it is difficult to assess the success of the molecular karyotypes recreation if mosaics were to represent a small proportion of those studied. A genetic burden by phenotype association test was then used and genetic variants were weighted based on their allele frequency and proximity for causality (Corbitt et al., 2019). A 13-fold increase risk of bicuspid aortic valve was associated with hemizygosity of *TIMP1* combined with deleterious variants in *TIMP3* (Corbitt et al., 2019).

DNA obtained from study 4 has now been submitted for ES in order to investigate the X chromosome as a whole and its interaction with autosomal genes by using TS as a model. It is hoped that the more causal effect of exome variants will counteract the power related issues experienced of a small sample size. Furthermore, there is a logic to combining data sets from study 4 and the future exomes data to examine both intronic and exome variants.

Study 4 provided a springboard for TS-related genomic research at UCL and UCLH. Study 4, will continue to actively recruit participants for the foreseeable. Whilst study 4 was underpowered conducting another array study so to combine the data with that of study 4 to improve power is not unjustified. In the meantime, the genotype data from study 4 should not go unanalysed as it would be interesting to revisit the current data set and explore the variants T1DM-associated or autoimmunity in order to really understand the potential genetic underpinnings of TS-associated DM.

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Appendices

Minor Corrections

Original page number and minor corrections are shown in red.

Page 2

I, Antoinette Cameron-Pimblett, confirm that the work presented in this thesis is my own, extending to laboratory work which I conducted myself. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. The molecular analysis in this thesis was supported in part by a Wellcome Trust Senior Research Fellow in Clinical Science grant awarded to Professor John Achermann (209328/Z/17/Z) and The International Fund Congenital Adrenal Hyperplasia. Several publications have been produced as a result of this work. Published content has been stated in each of the relevant sections and chapters. Publications have also been included adhering to University College London regulations.

Page 42

Obesity is a global health issue, defined by the World Health Organisation as a BMI ≥30kg/m². Hanew et al., observed obesity prevalence using BMI across 3 age groups in those with TS who had received GH treatment (n= 492) compared to the general female population.

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The Mosteller equation was used to calculate BSA; $\sqrt{(height(cm)\, x\, weight(kg)}/3600$. Female sex hormone profiles and AMH can be used to represent ovarian function. However, these tests are rarely performed other than at the initial investigation of patient symptoms and were therefore not examined as part of this work.

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The clinics at UCLH have records of 782 women with TS of whom 20 (2.5%) declined consent. For 762/782 individuals, the inclusion criteria for this report were a confirmed diagnosis of TS and a recorded TS karyotype. The original karyotype was not always available and therefore repeated or retrieved from local cytogenetic centres; St Thomas and Guys, Northwick Park, and Great Ormond Street hospitals. Karyotype was not available or retrievable for approximately 22% of the cohort. Karyotypes were available in 656 (78.1%) individuals.

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Table 4.2 summarises the paediatric cohort outcomes for each karyotype subgroup. Post-hoc analysis used to compare the outcomes for each outcome to that of 45,X.

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Further future work is needed to identify how many IGT women with TS go on to develop DM in order to refine how frequent OGTT should occur. As well as

research regarding whether or not IGT/DM risk is negated by weight management as it does in the general population.(Wilding, 2014)

Figure 6.1

The graph shows the age of onset for Turner Syndrome-associated Diabetes mellitus and includes newly diagnosed cases as part of study 3. Included is the published data on the age of onset of those with T1DM and T2DM as a means of comparison (Freeborn, Dyches, & Roper, 2017).

Additional references

Freeborn, D., Dyches, T., & Roper, S. O. (2017). Lessons Learned From a Life With Type 1 Diabetes: Adult Perspectives. *Diabetes Spectr, 30*(3), 188-194. doi:10.2337/ds16-0032

Wilding, J. P. (2014). The importance of weight management in type 2 diabetes mellitus. *Int J Clin Pract, 68*(6), 682-691. doi:10.1111/ijcp.12384

Dear Antoinette,

Project ID: 15/0877 (Please quote in all correspondence)

IRAS ID: 184846

REC Ref: 16/LO/0682

Title: The Reproductive Life Course Project V1.0

Amendment: Substantial Amendment 1.5

Confirmation of Amendment Capacity & Capability

The UCLH/UCL Joint Research Office (JRO) acknowledges receipt of the above amendment and the following documents:

a.   REC approval letter dated 04/03/2019 and therein listed documents.

b.   HRA amendment approval email dated 04/03/2019.

The JRO has no objections to this amendment and the study may continue at UCLH.

You must ensure that you localise all patient facing documentation prior to consenting participants; this will be subject to random audit checks.

Please forward this email on to all relevant parties involved with this study at UCLH.

Please insert a copy of this email in your site file.

Yours,

Novin Zahedi Fard

JRO Amendments Officer

Joint Research Office, Research Management and Governance

1st Floor Maple House (Suite B), 149 Tottenham Court Road, W1T 7DN

University College London Hospitals NHS Foundation Trust

Postal Address: Joint Research Office, UCL, Gower Street, London WC1E

Email: n.fard@nhs.net

Tel: 0203 447 2102

London - Chelsea Research Ethics Committee

Research Ethics Committee (REC) Bristol Centre
Level 3, Block B
Whitefriars
Lewins Mead
Bristol
BS1 2NT

Tel: 020 7104 8044

> **Please note: This is the favourable opinion of the REC only and does not allow** the amendment to be implemented at NHS sites in England until the outcome of the HRA assessment has been confirmed.

04 March 2019

Prof. Gerard Conway
Consultant Endocrinologist
University College London Hospital (UCLH)
250 Euston Road
Dept. of Women's Health
2nd Floor North
NW1 2PG

Dear Prof. Conway

Study title:	**The Reproductive Life Course Project: a quantitative analysis**
REC reference:	**16/LO/0682**
Amendment number:	**05/12/2018 Version 1.5**
Amendment date:	**22 January 2019**
IRAS project ID:	**184846**

Approval was sought for the following:

1) Information has been provided regarding the type of genetic testing, collaborators and how the information generation shall be used.
2) We have made change to the current study timeline to account for the above testing and dissemination of the results.
3) We have provided a GDPR statement.
4) Expanded our current UK- wide pregnancy audit to include centres whom may not have the resources to conduct the audit and patient support groups to set up a "self- referral" system to the UCLH team.
5) Points of contact within the research team have also been changed due to the use of the new nhs.net system.

The above amendment was reviewed at the meeting of the Sub-Committee held on 28 February 2019 by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Discussion

The Committee reviewed the amendment and made the following comments:

The Committee requested that in the PIS the researchers were more explicit about anonymisation when it is first mentioned under 'What is needed for genetic testing', e.g. DNA samples and corresponding clinical details will be pseudonymised (coded) *to remove any information which would allow people outside the research team to identify you from the sample*.

The applicant amended the PIS accordingly.

The Committee accepted the response.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Notice of Substantial Amendment (non-CTIMP) [AmendmentForm_Jan]	05/12/2018 Version 1.5	22 January 2019
Participant consent form [RLCP_Consent_v3]	3	05 December 2018
Participant information sheet (PIS) [v5_RLCP_Participant Information Shee]	5a	20 February 2019
Research protocol or project proposal [v1.5_RLCP_Protocol]	1.5	05 December 2018

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

Working with NHS Care Organisations

Sponsors should ensure that they notify the R&D office for the relevant NHS care organisation of this amendment in line with the terms detailed in the categorisation email issued by the lead nation for the study.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our Research Ethics Committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

16/LO/0682:	**Please quote this number on all correspondence**

Yours sincerely

██████████████

PP
Mr Roger A'Hern
Chair

E-mail: nrescommittee.london-chelsea@nhs.net

Copy to: Ms Tabitha Kavoi , UCLH
        Ms Suzanne Emerton

London - Chelsea Research Ethics Committee

Attendance at Sub-Committee of the REC meeting on 28 February 2019

Committee Members:

Name	Profession	Present	Notes
Mr Roger A'Hern	Medical Statistician	Yes	
Miss Noor Mujahid	Cell & Gene Therapy Formulation Scientist	Yes	

Also in attendance:

Name	Position (or reason for attending)
Mr Paolo Buscemi	REC Assistant

Reproductive Life Course Project
Reproductive Medicine Unit
Women's Health
2nd Floor, North Wing
250 Euston Road
London
NW1 2PG

Email: Antoinette.pimblett@uclh.nhs.uk

Version 1
05/01/15

Hospital No: XXXX
NHS No: XXXX
DOB: XXXX

Address XXX

Dear XXXXX

Reproductive Life Course Project: (CLINIC CODE INSERT)

I am writing to you as a person attending UCLH for manage of your **(INSERT DIAGNOSIS)**. I would like to invite you to join a new initiative- The Reproductive Life Course Project. This is research project I am inviting you to take part. It requires nothing more than you to fill in a questionnaire at your next clinic visit.

The aim of the project is to reflect on the data we have collected during the years of our clinic and to add a questionnaire to gain more information about you and your health. We hope that the project will reflect all aspects of your condition including psychological and social outcomes.

An information sheet about the project and your involvement is enclosed for you to read before your next appointment. During your next appointment you will be able to discuss the project with your doctor and decide as to whether or not you would like to join.

For further information please or to have the relevant documents sent ahead of your clinical appointment contact Antoinette Pimblett: Antoinette.pimblett@uclh.nhs.uk ext number 75701.

Yours sincerely,

Professor Gerard Conway
Consultant Endocrinologist
Hon Professor of Clinical Medicine UCL

University College London Hospitals NHS

NHS Foundation Trust

Reproductive Medicine Unit
Women's Health Division
North Wing, 250 Euston Road
London NW1 2PQ

Version Number: 5a
Participant Identification Number:
Date: 20/02/2019

The Reproductive Development

LIFE COURSE PROJECT

PARTICIPANT INFORMATION SHEET

Why have I been invited?

You have been identified to as an adult aged 18 or above, attending clinics at UCLH for the management of Disorders of Reproductive Development.

Background

Disorders of Reproductive Development (DRD) are a large group of disorders predominately affecting the endocrine system which occur up to 1 in every 500 people. Most DRD's have a known genetic background such as Klinefelters Syndrome, whilst other conditions such as Primary Ovarian Insufficiency do not have a strong genetic basis.

Associated with many of these disorders are co-morbidities such as hypothyroidism, osteoporosis (thinning of the bones) and fertility issues (i.e. sub- fertility). In order to record the natural history of the DRD's this study aims to capture health events over the life course of individuals and identify predictive factors that contribute to adverse outcomes. Experts therefore recommend that individuals with a DRD are reviewed in an outpatient clinic on a yearly basis to regularly monitor aspects of their health, such as blood pressure, thyroid blood tests, and bone density, and ensure any problems are detected and managed at the first possible opportunity.

There is potential for the medical information that is recorded during visits to these clinics to be compiled and analysed on a large scale, providing extremely valuable information about the medical, psychological, and social problems affecting individuals with DRD.

This study has been reviewed and given a favourable opinion by London- Chelsea Research Ethics Committee.

University College London Hospitals **NHS**

NHS Foundation Trust

What is the purpose of the study?

Most of the information currently known about the health and wellbeing problems affecting individuals with a DRD has been obtained from research involving small groups, over short periods of time.

The primary aim of the Reproductive Development Life Course Project is to expand on this current knowledge base, by examining the genetic causes, nature and frequency of major health events experienced by a large group of individuals with DRD, over a much longer time period. We hope to find out new information about the health problems experienced by individuals with DRDs, and publish it for the reference of other doctors, researchers and patients across the UK and around the world.

What is involved?

A.   Review of medical records

The first part of the study is carried out by members of the research team. They will obtain the medical records (both 'paper' and 'electronic' versions) of consenting individuals in order to extract relevant information, which will then be entered into a secure electronic database and analysed.

The information that will be extracted includes:

- Details of diagnosis (age, reason and genetic test results).
- Puberty, fertility and hormone replacement history.
- Other relevant medical problems or procedures (such as high blood pressure and thyroid gland dysfunction).
- Psychological wellbeing history such as depression, anxiety and anti-depressant use.
- Routine clinic visit data:
    - Height, weight and blood pressure.
    - Current hormone replacement and other medications taken.
    - Blood test results.
    - Bone density measurements.

B.   The Reproductive Development Life Course Questionnaire

Individuals who consent to take part in the study will be invited to complete a questionnaire at their next routine outpatient clinic appointment. The questionnaire has been designed to provide the research team with additional useful information that cannot always be found in medical records. The questionnaire will be in the form of a booklet and will take approximately 25 minutes to complete.

The questionnaire will cover the following topics:
- Physical health (including experiences with puberty, hormone replacement and fertility).
- Psychological well-being (including short screening questions designed to detect signs of low-mood and anxiety).

- Education, work and interactions with other people (including questions about family-life, partnership, bullying, and experiences in the outpatient clinic).
- A short review of recent physical activity, and food and drink intake.
- A short review of romantic and sexual experiences.

C. Genes and Genetic Testing

Your DNA contains a genetic code that is unique to you. A gene is a defined sequence of DNA that codes for a protein that is important for human functioning. Together, all of the genes that make proteins are referred to as the "exome". The "genome" refers to all parts of a person's DNA – both the parts that code for proteins and the parts that do not code for any proteins.

Many human clinical conditions or problems can be explained by genetic differences between individuals. It is thought that several patients with a DRD may have an underlying but as of yet undetected genetic diagnosis that accounts for their condition. Genetic testing refers to tests that aim to find out whether a person is carrying genetic changes that cause their condition. All consenting participants of this study will be invited to partake in genetic testing, with the hope of identifying changes that can account for DRDs.

What is needed for genetic testing?

Consenting subjects will be invited to give a blood sample. The blood sample will be taken by UCLH staff during a routine clinic visit and DNA extracted from it. DNA samples and corresponding clinical details will be pseudonymised (coded). Pseudonymised means that *any information which would allow people outside the research team to identify you from the sample will be removed*. Samples will then be transferred to a commercial laboratory and/or university-affiliated partners (eg. UCL GOS Institute for Child Health). DNA will be analysed using various techniques including:

- Single gene testing, where DNA is analysed for a change in one particular gene
- Panel-based testing, where multiple genes are tested for simultaneously
- Whole exome sequencing, where all sequences of a person's DNA that code for proteins (ie., all of a person's genes) are analysed simultaneously
- Whole genome sequencing, where all of a person's DNA is analysed simultaneously, both the parts that code for proteins and the parts that do not.

All of these DNA sequencing techniques can usually be performed on just one blood sample. A second sample is very occasionally required to verify findings. Very occasionally other cells may be analysed, such as skin cells, saliva or cells taken with a swab from inside the cheek.

Will I find out the results?

During the course of this research project we may identify genetic changes in you that may explain your clinical condition. If this happens we will ask you at your next clinic appointment if you would like to hear the result. We will also feed back at your request the overall study research findings. However, we will **not** feed back any genetic alterations in your DNA that are a by-product of this research, even if even if it may have implications for your health. This is because the genetic techniques used in this study are not suitable for diagnostic testing outside of the clinical conditions for which they are designed to investigate.

D. Future additional investigations

Consenting men and women will be able to take part in additional activities at a later date. These activities are not currently part of routine care, but may also provide useful information regarding the health outcomes for individuals with DRD. Examples include a more detailed measurement of body fat levels, ultrasound scans of the aorta or blood vessels, a more in-depth food intake interview and a psychological interview. This shall also include consent for the research team to track mortality data through death certificates should participants pass away.

What will taking part involve for me?

Part 1: You will have a meeting with a member of the research team who will explain the study to you and will be able to answer any questions you may have. If you agree to participate, you will be asked to sign a consent form. A letter shall also be sent to your GP informing them of your participation in the study.

Part 2: A member of the research team (such as a doctor or a specially trained research assistant) will obtain your medical records, and extract relevant information to be entered into and stored in a secure database. You do not need to be present for this part of the study.

Part 3: At your next clinic visit you will be invited to complete the questionnaire and give a blood sample for DNA extraction described above. A member of the research team will be present at the clinic to help you fill in the questionnaire before or after your doctor's appointment.

Part 4: At a later date, you may be asked if you would like to take part in additional activities or investigations which are not currently part of routine clinic follow-up (these are outlined in *section C* on the previous page of this document).

Do I have to take part in the study?

No, it is up to you to decide whether or not to take part. A decision not to take part will not affect the standard of care you receive at any time.

What are the potential benefits of taking part in the study?

By taking part in this study you would be contributing to research which will allow health professionals and patients to be better informed of factors contributing to the negative health outcomes that are frequently experienced by individuals with a DRD. Health professionals will then be able to modify their practice in order to optimise the health and wellbeing of current and future generations of individuals with a DRD.

University College London Hospitals **NHS**

NHS Foundation Trust

What are the potential risks or negative effects from taking part in the study?

Confidentiality

If you agree to take part in the study, your relevant medical files will be accessed by a small number of research staff who work closely with, but are not part of, your usual clinic team. These may include people from the Reproductive Life Course Project research team, and people employed by the UCLH NHS Trust who are involved in this research project. We may also consult with our commercial or university partners when drawing conclusions regarding potential results. Other governing bodies such as regulatory authorities may perform audits from time to time in order to ensure the teams are following Good Clinical Practice and trust practices.

Your clinical health data will be extracted from your medical notes and held in a database on the secure computer servers at University College Hospital. Your questionnaire will be pseudonymised (coded) then stored at a secure site on the UCH campus. All 'paper' and 'electronic' documents related to the study will only be accessible to approved members of the research team.

Your DNA sample and its corresponding clinical data will first be pseudonymised (coded). The code for this will be held on a database on a computer at UCLH using a secure NHS server. Pseudonymised (coded) DNA will be stored in secure fridges at UCLH. Samples of DNA may be transferred securely to commercial laboratories and/or to university affiliated partners (e.g. UCL GOS Institute of Child Health) for sequencing and analysis. Once analysis is complete, any remaining DNA will be securely transferred back to UCLH and stored in the secure fridges for up to five years after the study completion date. Once this time has been reached, the DNA samples are destroyed. Output from DNA analysis will be stored and archived on an NHS secure server.

The results of the study, including newly identified genetic findings and stories or statements written in the questionnaires, may be published for the reference of patients and medical professionals in scientific presentations and/or publications. No names or specific identifiers will be used. Genetic information produced by this research study may also be placed in a permanent electronic data archive indefinitely with no connection to your name or other personal identifier. Once published, this data is in the public domain and cannot be erased. Your identity cannot be connected to the genetic information produced by these studies by anyone apart from the Reproductive Life Course Project research team.

Challenging issues

Completing the Reproductive Development Life Course Questionnaire involves reflecting upon, and answering questions regarding issues that may be difficult for some participants to address. Some examples of potentially sensitive topics include bullying, psychological wellbeing and sexual function. If during the course of the study you feel that you would like to speak with someone regarding any distress or concern that has arisen from your participation in the study, you may contact a member of the research team Antoinette Pimblett (a.pimblett@nhs.net) or on phone 020 3456 7890 ext 75701, who will be able to arrange further assistance as required. If the matter is non-urgent, you may prefer to discuss it with your doctor at your next outpatient clinic.

GDPR Statement

UCLH ("we") is the sponsor for this study based in London. We will be using information from you and your medical records in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. UCLH will keep identifiable information about you for five years after study completion. The information will only be used for the purpose of health and care research, and cannot be used to contact you or to affect your care. It will not be used to make decisions about future services available to you, such as insurance

Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible.

What if I want to withdraw from the study?

Your participation in the study is voluntary. You may withdraw from the study at any time, without having to provide a reason. A decision to withdraw from the study will not affect the standard of care you receive.

Who should I contact for further information?

If you would like to speak to anyone about this study please do not hesitate to contact, Antoinette Pimblett (Clinical Research Assistant, Women's Health Division, University College Hospital) on a.pimblett@nhs.net (020 3456 7890 extn 75701). Alternatively, you may discuss any questions or concerns arising from the study with your doctor at your next TS outpatient clinic.

What happens next?

At your next outpatient clinic visit you will meet with a member of the research team, who will talk you through the study and answer any questions you may have. If you would like to participate, your written consent will be obtained and you be asked to complete the questionnaire before or after you see your doctor.

THANK YOU VERY MUCH FOR TAKING THE TIME TO READ THIS INFORMATION SHEET AND FOR CONSIDERING TAKING PART IN OUR RESEARCH STUDY.

University College London Hospitals NHS
NHS Foundation Trust

Reproductive Life Course Project
Reproductive Medicine Unit
Women's Health Division
North Wing, 250 Euston Road
London
NW1 2PG

PARTICIPANT CONSENT FORM

Name of Researcher _____

1. I confirm that I have read and understand the information sheet dated _____ (version_____) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected ☐

3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from regulatory authorities, by responsible people from the Reproductive Life Course Project research team, and by individuals from the UCLH NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my relevant records. ☐

4. I agree to give a blood sample for DNA extraction, sequencing, and analysis (including single gene, panel-based, whole exome, and whole genome sequencing approaches). I understand that my DNA sample will be pseudonymised (coded) on collection, may be transferred to a commercial laboratory and/or university-affiliated partners (eg. UCL GOS Institute for Child Health) for analysis, and may be used in studies up to 5 years after study completion in other research. ☐

5. I agree to my GP being informed of my participation in the study. ☐

6. I agree to being contacted at a later date in regards to taking part in optional additional investigations or activities related to the study such as a telephone interview. ☐

7. I agree to tracking of my mortality data (death certificate details). ☐

8. I understand that the information collected about me will be used to support other research in the future and my pseudonymised data may be shared with collaborating researchers. Specifically, my pseudonymised genetic data may be shared with university-affiliated partner institutions (UCL Great Ormond St Institute for Child Health) for the purpose of analysing my results. ☐

9. I understand that the genetic information produced may be placed in a permanent electronic data archive indefinitely with no connection to my name or other personal identifier. Once published this data cannot be erased. My identity cannot be directly connected to the genetic information produced by these studies by anyone apart from the Reproductive Life Course Project research team. ☐

10. I understand that I will be asked if I would like to hear any genetic result(s) newly identified during this research study that explain(s) or very likely explain(s) my clinical condition. However, I understand that I will not be told about any genetic alterations in my DNA that are identified as a by-product of this research. This will not affect my access to clinically approved genetic advice and genetic testing through other doctors caring for me in any way. ☐

11. I agree to participate in the above study. ☐

_____   _____   _____
Name of participant   Date   Signature

_____   _____   _____
Name of person taking consent   Date   Signature

When completed: 1 copy for participant
                              1 copy for research site file
                              1 copy for medical notes

Reproductive Life Course Project
Direct line: 020 3447 5701 / Ext: 75701
Switchboard: 0845 155 5000/ 020 3456 7890
Email: Antoinette.Pimblett@uclh.nhs.uk
Website: www.uclh.nhs.uk

Clinical Biochemistry

Oral Glucose Tolerance Test Procedure

GLUCOSE TOLERANCE TEST
WITH SAMPLES FOR INSULIN

Controlled Document: 336/DFT 10 GTT with samples for insulin test	Prepared by: Dr Gill Rumsby, Dr Anne Dawnay
Version: 4	Dr G Conway, Dr S Baldweg
Issued June 2009. Reviewed biennially.	Authorised by: Dr Gill Rumsby
Date of next review: February 2017	

Purpose

To clarify the aetiology of obesity, particularly the obese child*, where the differential diagnosis includes

- Simple obesity
- Cushing syndrome (although other tests are preferred for this diagnosis)
- Prader-Willi syndrome
- Lawrence-Moon-Biedl (LMB) syndrome
- Insulin resistance/acanthosis nigricans

*A separate protocol is available on Inform for use by paediatric endocrinology

Summary

Blood glucose, and blood insulin, following an oral glucose load.

Prior Conditions

Not diabetic, where diabetes is already diagnosed if either one of the following are present

- Fasting venous plasma glucose of ≥7.0 mmol/L on more than one occasion; or once if symptomatic.
- Random venous plasma glucose of ≥11.1 mmol/L on more than one occasion; or once if symptomatic.

Precise criteria are given by Diabetes-UK (2000) in conformance with WHO guidelines.

Prior Arrangements

Stop all medication, including steroids, likely to affect glucose tolerance. The patient must remain on a normal diet, containing a generous proportion of carbohydrate.

The 75g glucose load will be given as

POLYCAL, available from NHS Supplies (Product code; ABX075 Nutritional supplement carbohydrate liquid ready to drink neutral 200ml Tetra pack Polycal 18882).

or

 Liquid Maxijul (SHS International, Product code 0209).

Dose:

Adults: 113mL POLYCAL or liquid Maxijul, which must be measured out by the user.

Children: 2.6mL POLYCAL or liquid Maxijul per kg body weight (maximum 113mL).

The UCLH Trust approved glucose meter should be prepared for patient testing. Note this device must only be used by a trained member of staff. For full details of its use see the Standard Operating Procedure available on file in Phlebotomy and electronically on Insight (select tab Policies and Procedures, search for glucose meter).

Special Precautions

None

Procedure

No food should be taken from 8pm on the night before the test; but water is allowed. The test is performed in the morning. During the test, the patient should remain at rest and refrain from smoking, eating, or drinking anything except water.

Download the procedure-specific biochemistry request form from the Dynamic Function Test page of the Clinical Biochemistry intranet site.

Complete patient details on the form and, following the commencement of the test, write the clock times on the form and blood tubes as samples are taken.

In the fasting state, take Basal blood for glucose (grey = fluoride/oxalate tube) and insulin (gold top, serum).

Measure the blood glucose on the glucose meter. Where the metered glucose result is 10 mmol/L or higher:

- the oral glucose load should not be given.
- the remainder of this procedure should not be used.
- Send the fasting blood glucose and insulin samples to the laboratory immediately with a note of the meter result.
- Explain to the patient that the test will not continue because of the high fasting result.

(Note: the metered cut-off at 10 mmol/L is chosen to be definitely higher than the diagnostic cut-off for diabetes at 7.0 mmol/L, and is at a level which suggests that an oral glucose load could cause an undesirably high plasma glucose.)

Otherwise, continue as follows.

The POLYCAL or Maxijul (dose as above) should be drunk within 5 minutes with a glass (200mL) of water. Further water is permitted as desired.

Complete the collection of samples according to the Table:

Time	Glucose	Serum Insulin
Basal	✔	✔
30min	✔	✔
60min	✔	✔
120min	✔	(✔)

Where the Table shows **(✔)** the sample collection is optional. To maintain a complete set, at least label a tube and send it empty to the laboratory.

N.B. Insulin is labile in blood at room temperature. Rapid transport to the laboratory is required. It is acceptable to keep all the samples together i.e. send after the 120 min time point, but further delay is inadvisable.

Interpretation

POLYCAL and Maxijul are a solution of polymers (maltodextrin) which require digestion before absorption. In cases of malabsorption, the rise in blood glucose may be delayed but this test should not be used to diagnose malabsorption.

The blood glucose results may be interpreted as for a normal Glucose Tolerance Test.

Table

	Time	Glucose (mmol/L)
Impaired Fasting Hyperglycaemia (IFG)	Basal	6.1 – 6.9
Impaired Glucose Tolerance (IGT)	Basal	<7.0
and	120min	7.8-11.1
Diabetes (DM)	Basal	≥7.0
or	120min	≥11.1

The serum insulin results should be interpreted against the concurrent glucose results. Further interpretation of insulin is not given here.

Haemolysis can markedly affect insulin results. Any samples which are haemolysed will be cancelled by the laboratory

Reference

WHO Expert Committee on Diabetes Mellitus (revised 1999), WHO, Geneva, as implemented by the Diabetes-UK (Jan 2000).

http://www.who.int/diabetesactiononline/diabetes/basics/en/index4.html