The cytotrophoblastic shell and complications of pregnancy

[1]Graham J Burton and [2]Eric Jauniaux

[1]Centre for Trophoblast Research, Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge, UK

[2]Department of Obstetrics and Gynaecology, University College London Hospitals and Institute for Women's Health, University College London, London, UK.

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Address for correspondence:

Professor G.J. Burton,

Physiological Laboratory,

Downing Street,

Cambridge, CB2 3EG

UK

Email: gjb2@cam.ac.uk

Tel: +44 1223333856

Abstract

Many complications of pregnancy have their pathophysiological roots in the early stages of placentation. Impaired trophoblast invasion and deficient remodelling of the maternal spiral arteries are a common feature. While malperfusion of the placenta may underpin cases of fetal growth restriction and early-onset pre-eclampsia, the mechanistic links to spontaneous miscarriage, pre-term labour and premature rupture of the membranes are less obvious. Here, we speculate that formation of a well-developed cytotrophoblastic shell at the maternal-fetal interface is crucial for pregnancy success. Initially, extravillous trophoblast cells differentiate from the outer layer of the shell in contact with the endometrium. Impaired development may thus contribute to reduced invasion and deficient remodelling. In addition, the extent of the shell influences the timing and spatial configuration of onset of the maternal arterial circulation. A thin and fragmentary shell results in premature and disorganised onset, leading to spontaneous miscarriage. In less severe cases it may predispose to haemorrhage at the interface and formation of intrauterine haematomas. If pregnancy continues, these haematomas may act as a source of oxidative stress, promoting senescence and weakening of the membranes, and stimulating inflammation in the uterine wall and premature contractions. Formation of the shell is dependent on proliferation of cytotrophoblast progenitor cells during the first weeks after implantation, when the developing placenta is supported by histotrophic nutrition from endometrial glands. Hence, we propose the fitness of the endometrium prior to conception, and the peri-conceptional dialogue between the endometrium and the trophoblast is critical for avoidance of later complications of pregnancy.

Introduction

The placenta is key to a successful pregnancy and the life-long health of the offspring (1). In the human, placentation is a highly invasive process and more complex than in most other mammalian species. At the time of implantation the conceptus embeds into the superficial endometrium, and during the first and early second trimesters a sub-population of trophoblast cells, the extravillous trophoblast, migrate in large numbers into the wall of the uterus. Under normal conditions these cells reach as far as the inner third of the myometrium, a phenomenon referred to as 'deep placentation' (2). The invasion is associated with remodelling of the maternal spiral arteries, a process in which the smooth muscle and elastic material in the walls of the vessels is replaced by inert fibrinoid material (3). As a result, the vessels dilate, and remodelling ensures a constant high volume, low velocity maternal blood flow to the placenta (4). Deficiencies in deep placentation and arterial remodelling have been linked to a spectrum of complications of pregnancy (2, 5). Whilst it can be appreciated how some complications, such as growth restriction, early-onset pre-eclampsia and late spontaneous miscarriage, may arise through differing degrees of malperfusion of the placenta, it is more difficult to envisage a mechanistic link with pre-term rupture of the membranes and pre-term labour. Uteroplacental ischaemia has been invoked in the causation of the latter, with the suggestion of activation of the renin-angiotensin system in the fetal membranes (6).

Here, we propose an alternative hypothesis to link the pathophysiology of this spectrum of placentally-related complications of pregnancy. Central to the hypothesis is the

correct formation of the cytotrophoblastic shell, the layer that represents the interface between the maternal and placental tissues during early pregnancy.

The cytotrophoblastic shell

Initial growth of the placenta is prolific, and considerably in advance of that of the embryo. Shortly after implantation the chorionic sac is covered over its entire surface by a mass of developing villi, each consisting of a core of mesodermal cells and a bilaminar trophoblastic epithelium composed of an outer layer of syncytiotrophoblast and an underlying layer of progenitor cytotrophoblast cells. The syncytiotrophoblast is absent at the distal ends of the villi where they make contact with the decidua, and instead the cytotrophoblast cells form an elongated mass of cells referred to as a cytotrophoblast cell column. At their furthest extent these columns make contact with the decidua basalis, and in doing so spread laterally and merge with neighbours to form the cytotrophoblastic shell.

One of the most comprehensive descriptions of the shell was provided by Hamilton and Boyd (7), who had the opportunity to study 37 specimens ranging from 11-12 days to 90 days post-fertilisation (embryonic crown-rump length of 60 mm). These authors described the shell as being 'thick' at 14-18 days, 'attenuated' at 20-30 days, and 'markedly thinned' from the 10 mm stage, 37-38 days post-fertilisation, onwards. We have been able to review some of the same specimens from day 26 onwards contained within the Boyd Collection. At 26 days, the shell extends across the placental bed and continues beneath the decidua capsularis, forming an almost complete layer, 5-10 cell thick, that constitutes the fetal-maternal interface around the implanted conceptus (Figure 1). Anchoring villi attaching to the placental side of the shell by

cytotrophoblastic cell columns are numerous at this stage, and closely approximated together. By day 40 post-fertilisation, the shell is variable in thickness, remaining several cells thick where cell columns are attached, but gradually reducing to a single cell layer in the intervals between the columns (Figure 2). Expansion of the chorionic sac means that the distance between the cell columns increases, and so in later specimens, the shell becomes discontinuous, persisting only where cell columns are attached (7-9). In the intervals, fibrin is laid down at the fetal-maternal interface, generating Nitabuch's stria. Later in gestation, the remnants of the shell and Nitabuch's stria are incorporated into the developing basal plate (10).

The cells of the shell are derived from the proliferative zone at the proximal end of the cytotrophoblast columns (Figure 3). Many studies have shown that mitotic figures and immunohistochemical markers of cell division are only seen in cytotrophoblast cells either in contact with the villous basement membrane or within a few cell layers of it (11, 12), leading to the concept that this represents a stem cell niche (13). Cytologically the cells appear undifferentiated, and their cytoplasm contains only a small amount of endoplasmic reticulum and Golgi bodies (14). As the cells move away from the basement membrane they undergo differentiation involving Notch signalling pathways (15), and enter a post-mitotic state (16). Glycogen progressively accumulates within the cytoplasm, and consequently the cells often appear conspicuously pale in histological sections as the deposits are eluted during routine fixation. Intermediate filaments become abundant, and numerous desmosomes link the cells (14). The amount of endoplasmic reticulum increases, and extracellular matrix material begins to be seen in the interstices between the cells. The columns and the shell are continuous with one another (Figures 1 and 3), and cells within the shell retain a similar rounded

morphology surrounded by matrix-type fibrinoid (17). More extensive deposits of fibrinoid are seen at the interface between the shell and the maternal tissues, where they form an irregular and commonly incomplete layer referred to as Nitabuch's stria (Figure 3). This marks the future plane of separation of the placenta at the time of delivery.

At present, the factors regulating cytotrophoblast cell proliferation are not fully understood, but two facets of the intrauterine environment during the first trimester are thought to be important. First, is the histotrophic support from the endometrial glands. The endometrial glands deliver carbohydrate and lipid-rich secretions into the intervillous space during early pregnancy (18), and these secretions contain powerful mitogenic growth factors, including epidermal and fibroblast growth factors (19). Application of such growth factors to first trimester villus explants results in increased proliferation of the cytotrophoblast population (20, 21). Indeed, in many species there is evidence that the trophoblast is able to signal to the glands and upregulate the expression of growth factors (22), and in this way stimulate its own development. Experimental evidence for such a mechanism operating in the human is lacking, although the key components appear to be in place (23). In addition, it is well-recognised that the gland cells adopt a characteristic hypersecretory morphology during early pregnancy, the Arias-Stella reaction (24). On the placental side, it is notable that the proliferative cells in the putative stem cell niche at the proximal end of the column immunoreact positively for the fibroblast growth factor receptor 2, and signalling from this receptor enhances expression of *CDX2* and *ELF5* (13). These genes encode two transcription factors that are essential for stem cells of the trophoblast lineage.

Second, a low oxygen concentration prevails within the developing placenta during early pregnancy (25), and this may favour proliferation of the cytotrophoblast progenitor cells (26). There may well be interactions between the two facets, for the levels of CDX2 and ELF5 drop sharply at the end of the first trimester (13), coinciding with the transition from histotrophic to haemotrophic nutrition and a three-fold rise in intra-placental oxygenation (25). Some proliferation may continue in the niche at the proximal end of a column, but the implication is that the proliferative potential of the trophoblast is greatly reduced during the second and third trimesters.

The importance of the cytotrophoblastic shell in normal pregnancy

The integrity of the shell is critical during the early stages of pregnancy for several reasons. It provides anchorage to the extracellular matrix of the maternal endometrium (9), but it is primarily its functions relating to onset of the maternal arterial circulation to the placenta that are the focus of this review. Firstly, it is the source of the extravillous trophoblast cells that are involved in the remodelling of the spiral arteries. Cells towards the outer surface of the shell undergo a partial epithelial-mesenchymal transition to form interstitial trophoblast cells (9, 27, 28). This transition is associated with a marked change in their morphology, for they adopt a spindle-like shape with a dark-staining nucleus (Figure 4) (8, 12). This transition is possibly induced by the higher oxygen concentration within the decidua with which they are in contact (25, 29), but may also be initiated by hormones and cytokines released by the decidual cells. Interstitial trophoblast cells migrate through the decidua and into the inner third of the myometrium where they fuse to form multinucleated trophoblast giant cells (30). Interstitial trophoblast are particularly numerous surrounding the spiral arteries, and their presence appears to be essential for vascular remodelling (8, 31). Increased rates

of apoptosis and reduced invasiveness of these cells have both been invoked as reasons for deficient remodelling of the arteries in cases of growth restriction and pre-eclampsia (12), but it is equally possible that a reduced supply of cells from the shell, and ultimately from the progenitor niche at the proximal end of the cytotrophoblast cell columns, might also contribute.

Secondly, when the advancing margin of the shell penetrating the decidua basalis encounters the distal portion of a spiral artery, trophoblast cells migrate down the lumen of the artery as endovascular trophoblast (8). These cells retain their rounded morphology and appear identical to those of the shell, although they do show immunoreactivity for CD56 that is not seen within the shell (31). The magnitude of this migration is sufficient to virtually occlude the spiral arteries during the first six weeks of pregnancy, restricting any flow into the intervillous space to a seepage of plasma through the network of narrow intercellular clefts (32). The clefts gradually expand and coalesce over the next few weeks, until free flow of arterial blood is established around 10-12 weeks of pregnancy (25, 33). Restriction of maternal arterial inflow is essential during early pregnancy to protect the developing embryo from exposure to the oxygen in the maternal circulation, and free radical-mediated oxidative teratogenesis (34, 35). Development of the shell assists by providing a source of endovascular trophoblast cells over a broad area, ensuring there is a sufficient supply to plug any maternal vessels encountered by the expanding placenta irrespective of their precise location. This will be the case in the central region of the implantation site where the shell is thickest (2). Towards the periphery the shell is thinner, and so the opportunity for plugging of the spiral arteries is less in these areas (Figure 5A). Hence, onset of the maternal circulation is seen preferentially in the periphery, and results in locally high levels of oxidative

197 stress as the villi display very limited antioxidant defences at this stage of development
198 (36). This stress is thought to induce villus regression and formation of the smooth or
199 free membranes of the definitive placenta, and may be considered physiological as it
200 occurs in all ongoing pregnancies.
201
202 Once the shell becomes fragmented from 40 days post-fertilisation (8 weeks of
203 pregnancy) onwards, the source of extravillous trophoblast cells must be principally
204 from the remnants located where the distal ends of cell columns make contact with the
205 decidua (Figure 2A). This spatial rearrangement will have little impact on plugging of
206 the arteries, as onset of the maternal circulation begins progressively from around this
207 time (35). Equally, interstitial trophoblast will continue to flow from the cell columns
208 and migrate through the endometrial stroma, homing in on the spiral arteries. Although
209 the cell columns shorten as gestation advances, cytotrophoblast cells remain
210 proliferative in the proximal progenitor niche until at least 16-20 weeks of pregnancy
211 (12). The number of cell columns may increase during pregnancy through subdivision of
212 the early anchoring villi, possibly facilitated by the faster expansion of the developing
213 basal plate in comparison to the chorionic plate (10). In addition, branching
214 morphogenesis of the villous trees may bring further villi into contact with the shell,
215 establishing new points of attachment (9).
216
217 **Impaired development of the cytotrophoblastic shell and complications of**
218 **pregnancy**
219 While developmental differences in the extent of the shell are related to local variations
220 in the timing of the onset of the maternal circulation in normal pregnancies, gross
221 impairment of its development is associated with the pathology of spontaneous

miscarriage. In 70% of these cases the shell is thin and fragmentary, leading to deficient endovascular trophoblast migration and incomplete plugging of the spiral arteries across the entire placental bed (37, 38) (Figure 5B). Onset of the maternal circulation is precocious and spatially disorganised, with massive entry of maternal blood resulting in overwhelming placental oxidative stress and secondary degeneration of the villous tissue (36). This effect is independent of the trophoblastic karyotype, and so we must look beyond the conceptus for a cause.

Normal pregnancy and miscarriage represent opposite poles of pregnancy outcomes, but is it possible that other placentally-related complications of pregnancy are associated with intermediate degrees of development of the cytotrophoblastic shell? Spiral arterial remodelling is also deficient in cases of growth restriction, and even more so in those with accompanying pre-eclampsia when obstructive arterial lesions may also be present (2, 39, 40), but to a lesser extent than what is observed in early pregnancy failure. These vascular changes likely also reflect reduced trophoblast invasion into and around the arteries, and so it might be expected that arterial plugging was less extensive in these placentas during early pregnancy. Consequently, onset of the maternal circulation may have been abnormal, both temporally and spatially. Currently, no data are available to support or refute this hypothesis, and future prospective studies are required to test the concept. However, the fact that placentas from pregnancies complicated by growth restriction often display irregular margins and excessive villous regression provides some circumstantial support (41).

Besides influencing timing of the onset of the maternal circulation, the extent of development of the shell may impact on the integrity of the maternal-fetal interface and

247 the adhesion between the two sets of tissues (9). The regression of around two-thirds of
248 the original villous mass of the early placenta creates an area of mechanical weakness in
249 the periphery where the spiral arteries are unplugged, leading potentially to bleeding
250 between the developing membranes and the decidua basalis at the end of the first
251 trimester (Figure 5B). This phenomenon is known clinically as threatened miscarriage,
252 and is the most common complication of human pregnancy.
253
254 Sub-chorionic haematomas are well defined on ultrasonic examination as crescentic
255 hypoechogenic areas between the placental membranes and the decidua. If the
256 haematoma expands to the basal plate of the definitive placenta it can lead to full
257 detachment of the placenta and a full miscarriage, which is observed in around 10% of
258 the cases within 48 hours of the first bleeding episode (42, 43). In the 90% of
259 pregnancies that continue, there is a 1.9-3.7 increased risk of premature rupture of the
260 membranes and pre-term delivery (43). The mechanistic link has not been fully
261 determined, but it has been postulated that if the pregnancy continues the clot of blood
262 lying against the membranes causes local oxidative stress (42). In particular, the
263 presence of free Fe^{2+} ions may stimulate the formation of the highly aggressive hydroxyl
264 ion through the Fenton reaction (44). Chronic exposure to reactive oxygen species can
265 cause cellular senescence, and this has recently been put forward as the final common
266 pathway for weakening and premature rupture of the membranes in response to
267 various stimuli (45). In addition, senescent cells secrete a cocktail of pro-inflammatory
268 cytokines (46), and this may lead to the induction of a sterile inflammatory response
269 within the uterus that results in pre-term delivery (47). Changes in maternal levels of
270 placental specific proteins (48, 49), and also of inflammatory cytokines (50) and

markers of oxidative stress (51) in women presenting with a threatened miscarriages support this concept.

If considered from the viewpoint of development of the cytotrophoblastic shell it is to be expected that the two sets of pathologies, namely early pregnancy failure, growth restriction and pre-eclampsia on the one hand, and pre-term premature rupture of the membranes and pre-term delivery on the other should show epidemiological associations, and also links to events during early pregnancy. This is indeed the case (43).

Future directions

Human early pregnancy is a difficult period to research, and development of the cytotrophoblastic shell that we propose to be critical is occurring before and shortly after pregnancy is manifested clinically. Data from other species indicate that the signalling dialogue between the conceptus and the endometrium is essential for upregulation of the secretion of growth factors that stimulate trophoblast proliferation, and hence likely formation of the cytotrophoblastic shell (22). Although recent data for the human indicate the importance of the endometrial secretome for implantation (52, 53), the full composition of the gland secretions and their impact during early pregnancy are not known. Uterine flushing at this time may not be ethical, and in any case may not accurately reflect the activity of the glands within the placental bed where local trophoblast interactions may influence gland activity. The derivation of endometrial organoids that faithfully replicate the transcriptomic profile of the glands and which respond to pregnancy hormones by upregulating expression and secretion of uterine milk proteins opens an important avenue for new research in this area (54, 55).

Overall conclusion

Each of the 'Great Obstetrical Syndromes' has many potential causes, some of which will be unrelated to trophoblast invasion, such as those of genetic or infective origin, whereas others will be associated with a failure of deep placentation. Focussing on formation of the cytotrophoblastic shell takes us one step earlier in the establishment of the pathophysiology of the latter cases, for the extravillous trophoblast differentiate from the surface of the shell abutting the maternal tissues. An insufficient pool of progenitor extravillous trophoblast cells within the shell will result in reduced endovascular invasion and inadequate plugging of the spiral arteries. At its extreme this can result in miscarriage (37, 38), but we speculate that less severe impairment may lead to intrauterine haematomas at the maternal-fetal interface. Such haematomas may render the membranes vulnerable to senescence and premature rupture, or stimulate inflammation in the myometrium and enhanced uterine contractility. Deficient interstitial extravillous invasion may also result in a reduced extent of arterial remodelling, leading to early-onset pre-eclampsia or growth restriction alone depending on the severity.

The principal implication of viewing the pathophysiology of these syndromes in this way is that formation of the shell, and in particular proliferation within the progenitor cell niches at the proximal ends of the cytotrophoblast cell columns, become of paramount importance. At present, little is known regarding the control of cytotrophoblast proliferation, but the unique first trimester intrauterine environment appears to be essential. Mitogenic factors secreted by the glands are likely to be critical (22, 23, 41), possibly in combination with the prevailing low oxygen concentration. Hence, some

cases of these syndromes may have their pathological roots in impaired endometrial function during the peri-conceptional period and early pregnancy, a view supported by genetic analyses of chorionic villus samples from women who went on to develop pre-eclampsia (56, 57). Further studies are required to test the hypothesis, but if proved correct then ensuring optimal endometrial function prior to conception should become a public health priority.

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The Boyd Collection of archival histological material is held by the Centre for Trophoblast Research (www.trophoblast.cam.ac.uk) at the University of Cambridge, and is available for viewing.

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Figure legends

Figure 1. Photomicrograph of a 26 day post-fertilisation placenta-*in-situ* specimen (H710) illustrating the cytotrophoblastic shell (CS) forming the maternal-fetal interface. The main illustration is taken from the area marked by the box on the low-power insert, towards the margin of the implantation site and the junction of the decidua basalis and decidua capsularis. Note the spaces (asterisk) within the shell that communicate with the intervillous space and the maternal vasculature. CCC, cytotrophoblast cell column. Stain, Masson's trichrome. Scale bar = 0.5 mm.

Figure 2. Photomicrographs of a 40 day post-fertilisation placenta-*in-situ* specimen (H673) illustrating the variable thickness of the cytotrophoblastic shell (CS) at this stage of gestation. A) At points of attachment of cell columns (asterisks) the shell remains thick, but in intervening areas it is very thin (arrows). B) Higher power view of the central area shown in A), illustrating the gradual reduction in thickness of the shell with increasing distance from a cell column. Stain, Masson's trichrome. Scale bars; A = 0.5 mm, B = 100 μm.

Figure 3. Photomicrograph of a 26 day post-fertilisation placenta-*in-situ* specimen (H710) illustrating how cells from a cytotrophoblast cell column (CCC) feed into the cytotrophoblastic shell (CS). Cytotrophoblast cells proliferate in a progenitor niche (asterisk) at the proximal end of a cytotrophoblast cell column, extending from an anchoring villus (AV). The columns spread laterally at their distal ends and merge with neighbours to form the shell. Note the deposition of fibrin (Nitabuch's stria) (arrowed) between the shell and the decidua (D). Stain, Masson's trichrome. Scale bar = 50 μm.

Figure 4. Photomicrograph of a 26 day post-fertilisation placenta-*in-situ* specimen (H710) illustrating the differentiation and migration of interstitial extravillous trophoblast cells from the shell. The cytoplasm of the cells within a cytotrophoblast cell column (CCC) and the cytotrophoblastic shell often appears empty as the high glycogen content is eluted during routine fixation. Cells near the maternal surface of the shell undergo a partial epithelial-mesenchymal transition, becoming darker staining and spindle-shaped (black arrow), and invade into the maternal tissues (white arrows) Immunostaining for cytokeratin 7 on equivalent age sections (insert) confirms the spindle-shaped morphology of many of the invading trophoblast cells. Stain, Masson's trichrome. Scale bar = 100 μm.

Figure 5. In normal pregnancies (A), extravillous trophoblast cells originating from the cytotrophoblast shell invade into the mouths of the maternal spiral arteries during the first trimester, preventing full arterial inflow into the intervillous space. Formation of the shell and plugging of the arteries is least in the periphery of the developing placenta where some inflow may occur, causing villus regression and formation of the smooth membranes. In pathological pregnancies (B), the cytotrophoblast shell is poorly developed. In the most severe cases this leads to early onset of the maternal arterial circulation to the placenta and miscarriage. If the pregnancy continues, there will be deficient spiral arterial remodelling due to inadequate extravillous trophoblast invasion. There may also be bleeding at the maternal-fetal interface and formation of an intrauterine haematoma (red), which may induce senescence in membranes and their premature rupture or an inflammatory response in the placental bed, increased uterine contractility and premature delivery. Adapted from (58).

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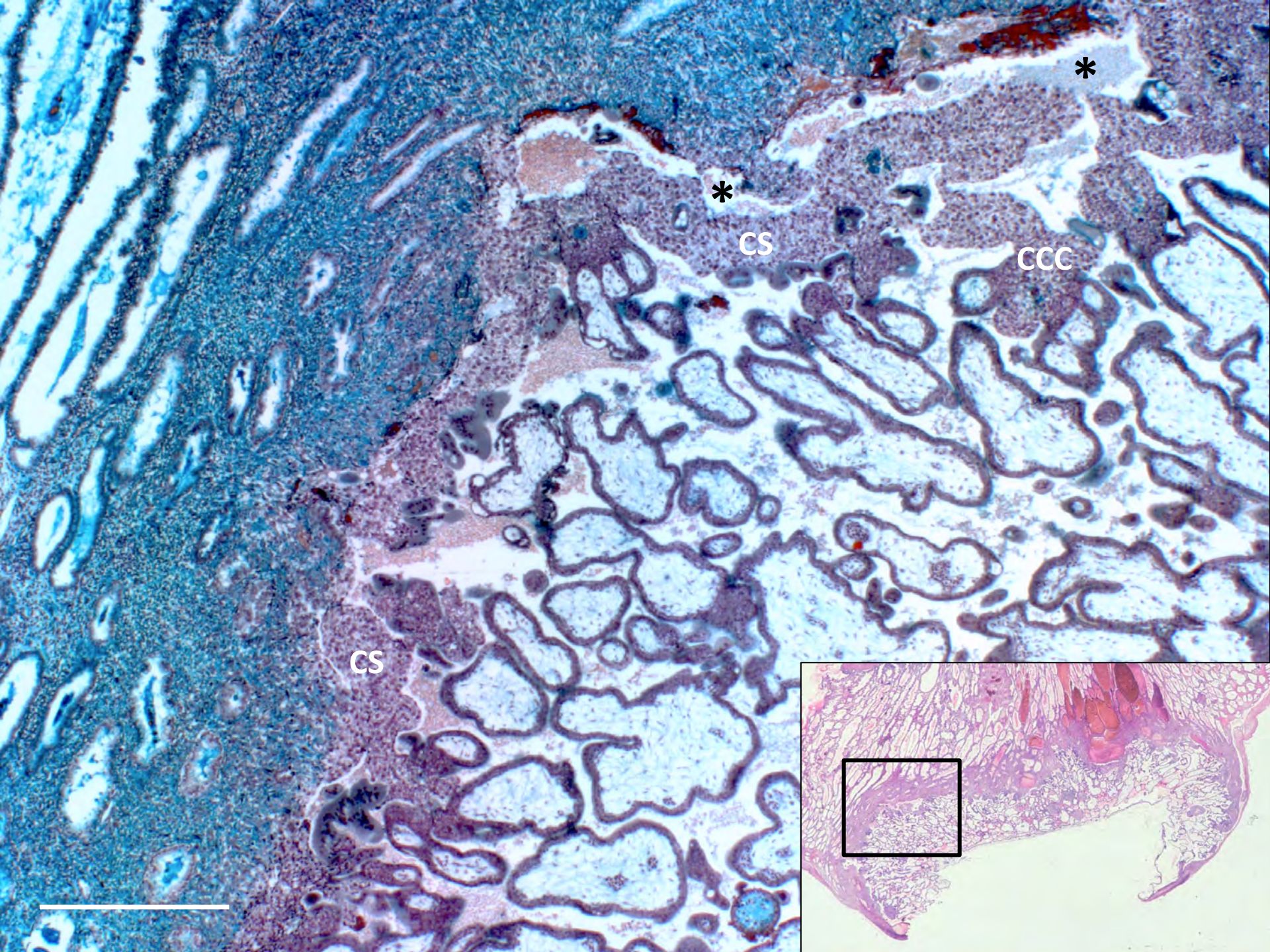
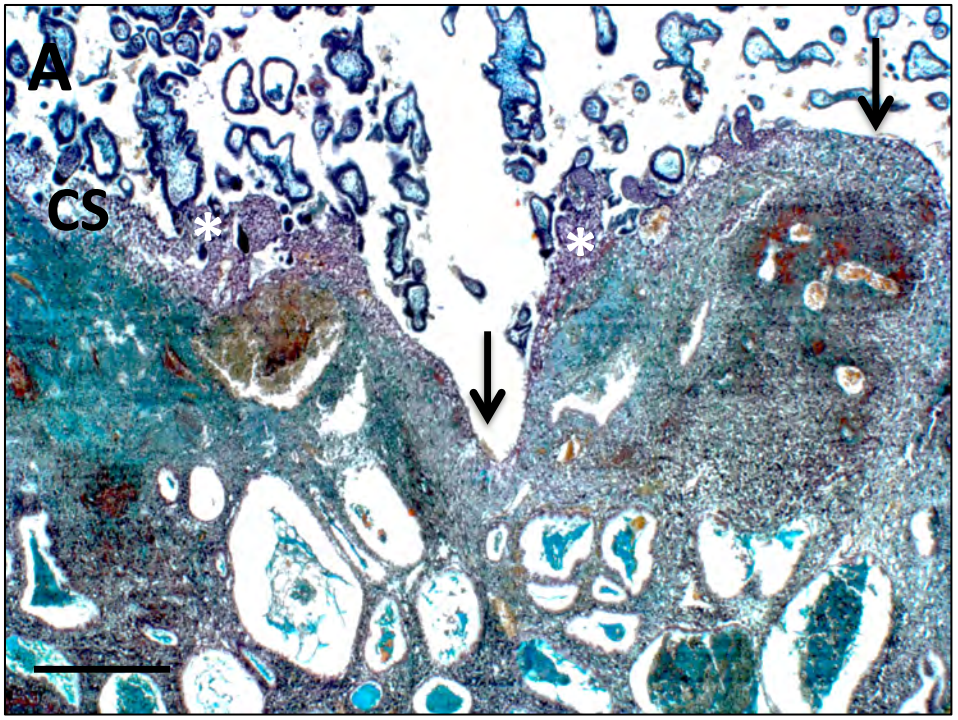

*
*
CS
CCC
CS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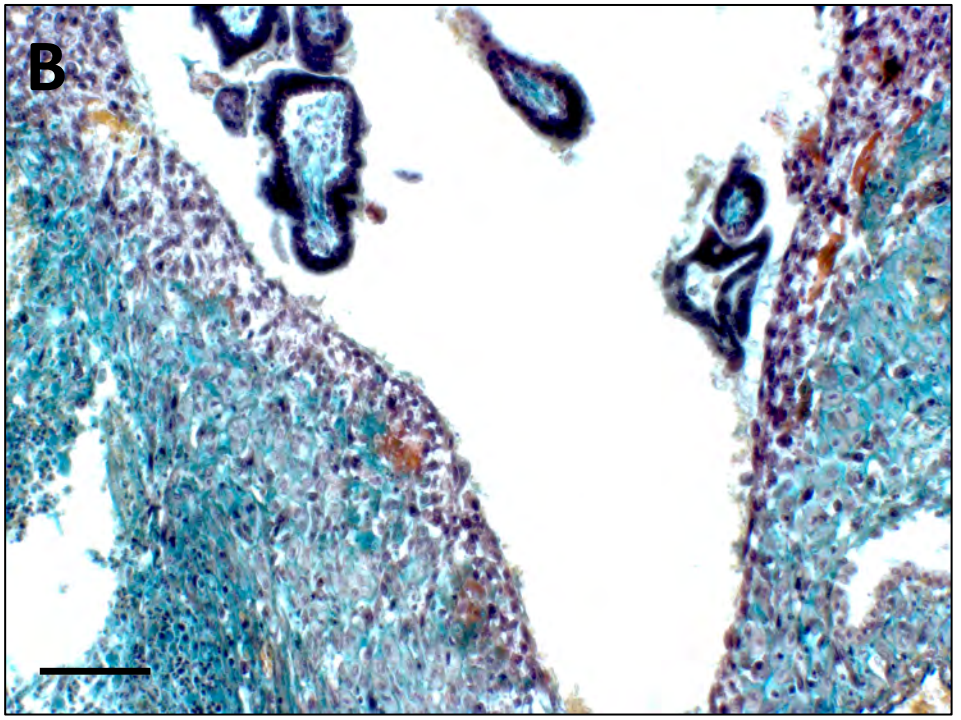
CS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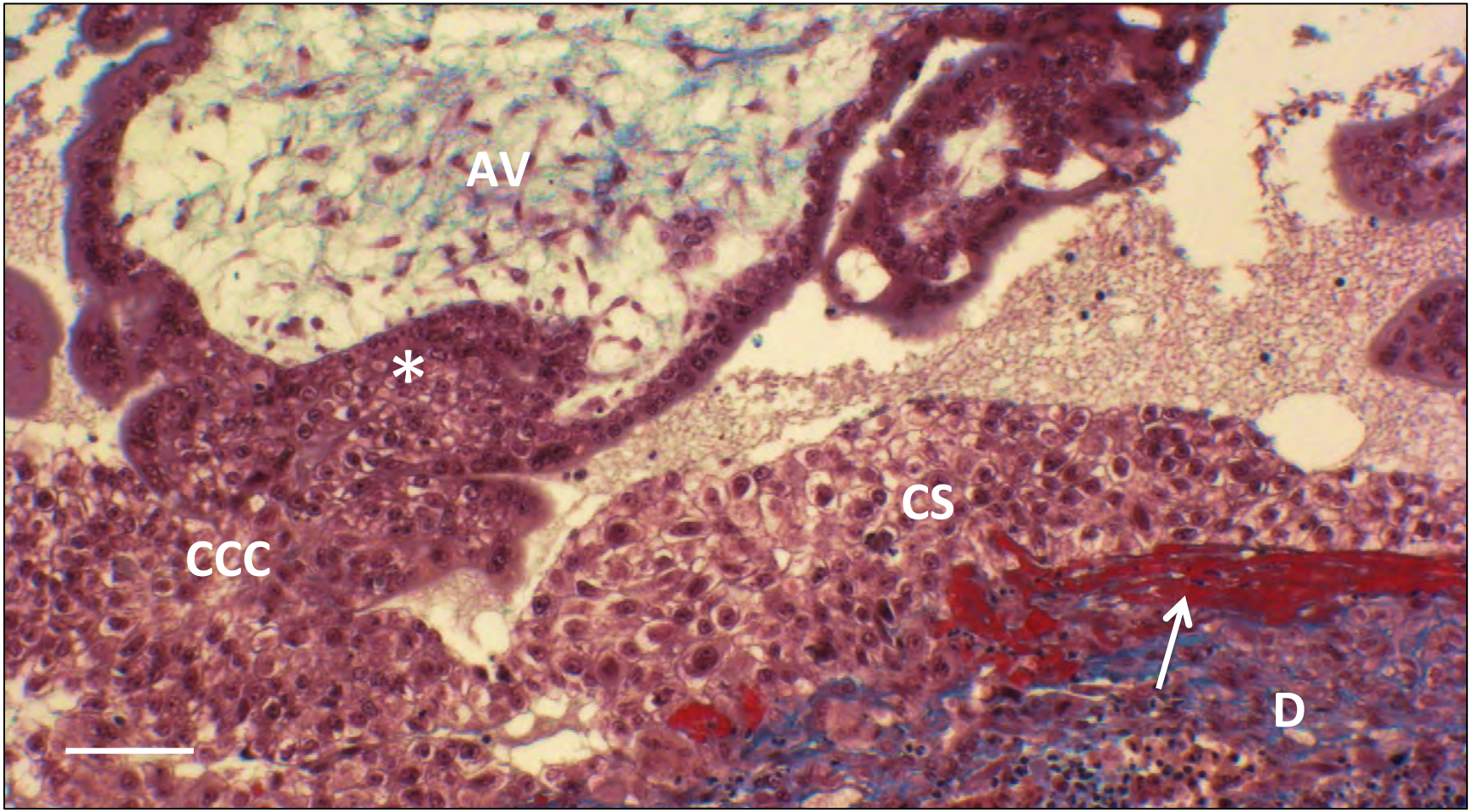
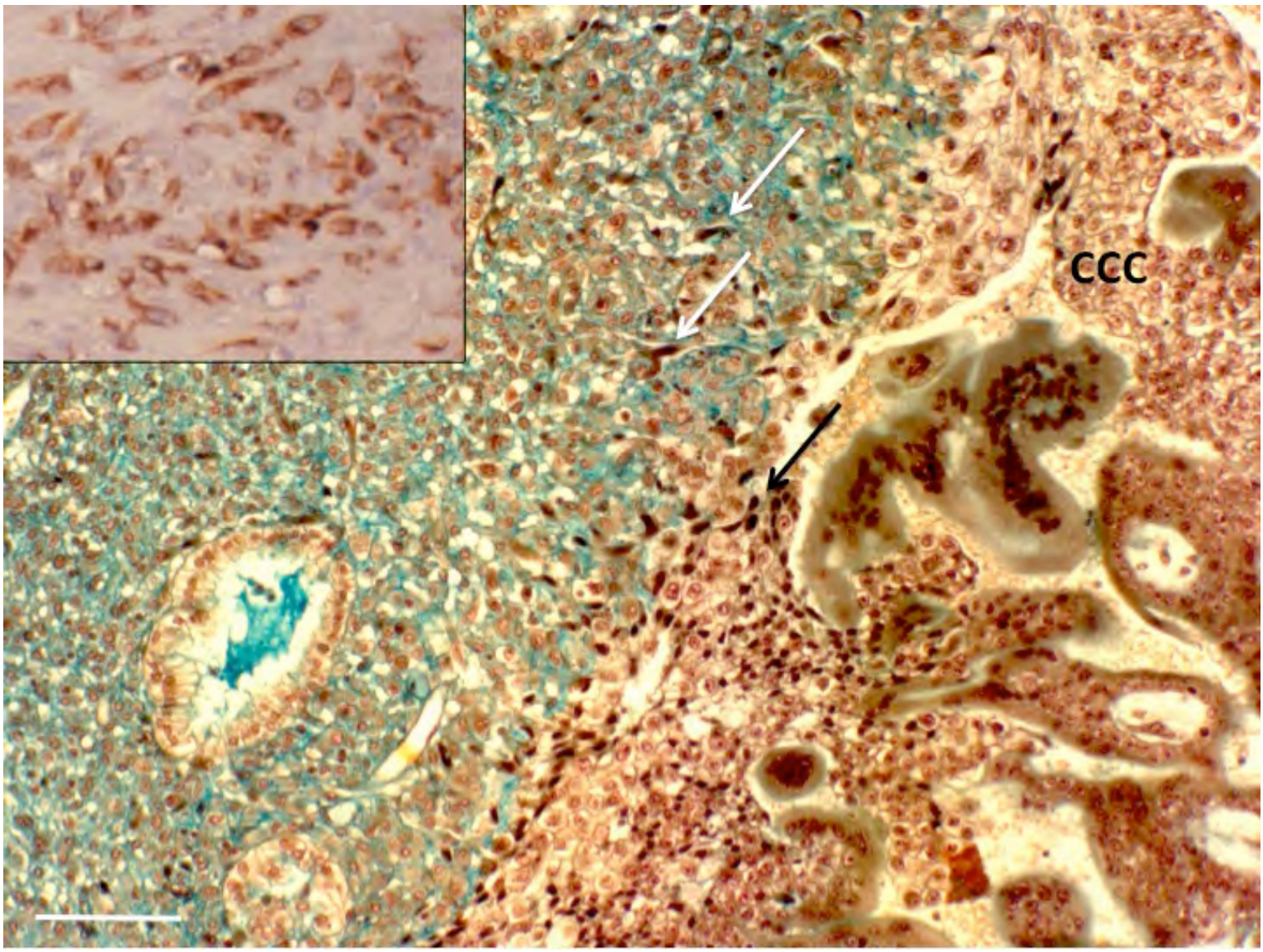

AV
*
CCC
CS
D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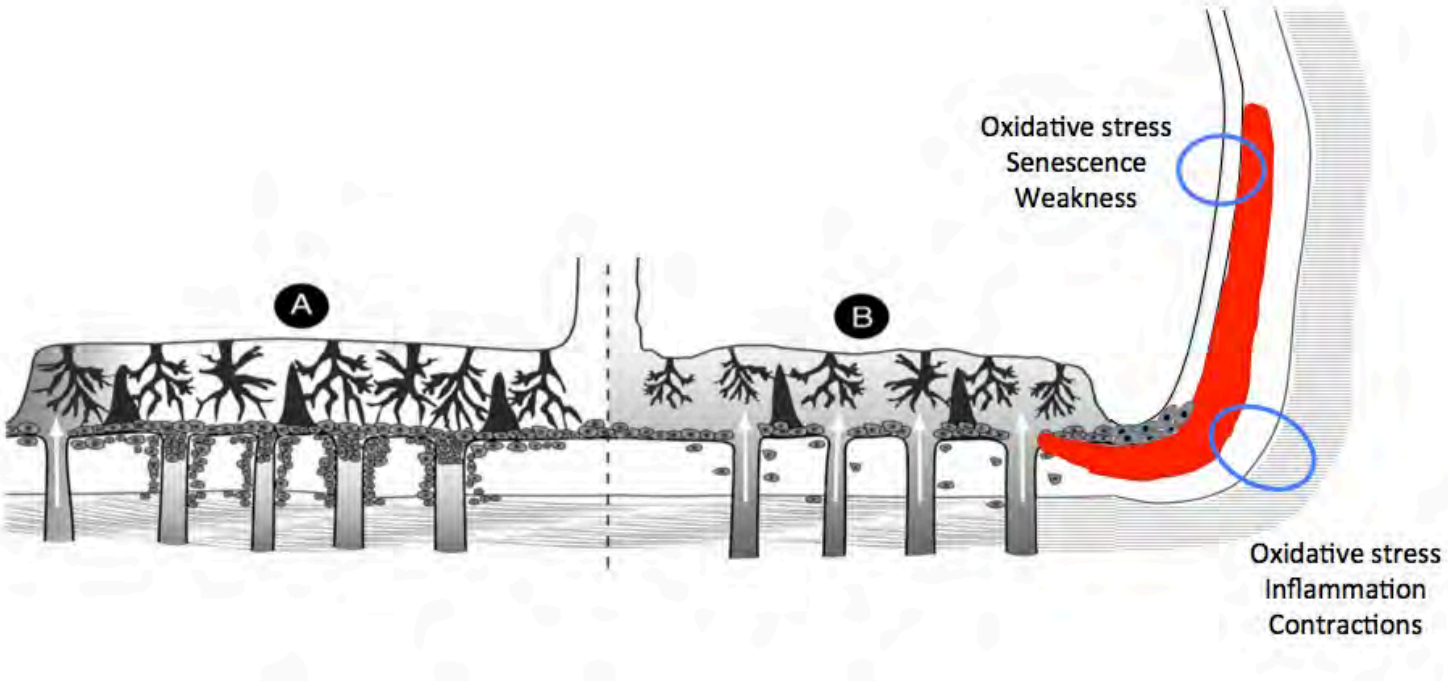
Oxidative stress
Senescence
Weakness
A
B
Oxidative stress
Inflammation
Contractions