Establishing Methods for the Detection of Fetal Microchimerism

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Gina Amy Bondi Knowles

Abstract

Cardiovascular disease (CVD) is a major contributor to both mortality and disability worldwide, with numbers of cases continuously rising. While CVD is commonly thought to primarily affect men, it is a killer amongst women as well. Despite this, women are strikingly underrepresented in research on cardiovascular health, likely leading to reduced quality in the treatment, diagnosis, and prevention of female CVD. This poses an urgent need for the acknowledgement and investigation of female-specific risk factors. Preeclampsia is correlated with an increased risk for later developing cardiovascular disease, even after adjusting for common risk factors such as obesity and high blood pressure. Preeclampsia is also associated with increased levels of fetal cells in maternal circulation during pregnancy, known as fetal microchimerism. These cells can harbor in maternal tissue, and we postulate that the fetal cells contribute to the increased CVD risk of women with a history of preeclampsia.

In the present work, methods for detecting the presence of fetal cells within maternal tissue through targeting the male-specific Y-chromosome, were developed. Digital droplet PCR (ddPCR), for the detection of male DNA, was performed on atherosclerotic tissue from 15 women and 1 man, used as a positive control. Fluorescent in situ hybridization (FISH), for spatial detection of male cells within female tissue, was validated using placental and decidua basalis samples, and implemented on atherosclerotic tissue from 4 postmenopausal women and 1 man.

Both ddPCR and FISH successfully demonstrated XY-positive cells in male atherosclerotic tissue. No male DNA was detected within female atherosclerotic tissue using ddPCR. Using FISH, a single XY-positive cell was detected in plaque from one female.

Further verification of fetal microchimerism detection in atherosclerotic tissue is needed through excluding the possibility of false positives, investigating larger areas of tissue, and increasing sample size. For ddPCR, sufficient DNA yield is necessary for fetal cell detection and thus a step that needs to be optimized. Further optimization of the FISH protocol may be carried out to yield improved and consistent fluorescent signals. Improvement of these methods as well as studying other cellular markers may elucidate the role of the ever-enigmatic fetal microchimerism in atherosclerosis and cardiovascular disease.

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1 Introduction

1.1 Cardiovascular disease

The term cardiovascular disease, abbreviated CVD, encompasses a set of disorders that affect the heart, brain, or their associated blood vessels. The main CVDs include coronary heart disease, cerebrovascular diseases, venous thromboembolism, and peripheral arterial disease. Coronary heart disease is characterized by the restriction of oxygen-rich blood to the heart due to plaque formation in the inner arterial wall. Further, this leads to myocardial ischemia, a condition in which the oxygen consumption of the heart exceeds its supply. The main symptom of this condition is angina pectoris, characterized by chest pain and discomfort. Over time, the artery wall may suffer plaque build-up, or the plaque may tear away. The latter causing thrombus formation at the rupture site. Both thrombus formation and plaque build-up can lead to pathologic blockage of blood to the heart, thereby causing necrosis and death of heart muscle cells. This is defined as a myocardial infarction and can be fatal [1].

Cerebrovascular diseases involve those that are caused by ischemia or bleeding of the cerebral blood vessels. If the flow of oxygen-rich blood to the brain is reduced, this will subsequently lead to the death of brain cells which is commonly known as a stroke. A transient ischemic attack, is a temporary blood flow restriction, less than one hour usually, and therefore does not result in any permanent tissue damage. In the case of subarachnoid hemorrhage, blood vessels on the surface of the brain may leak or burst, thereby bleeding into the area between the brain and the skull. This will in turn cause pressure on the brain, possibly leading to cerebral herniation and death. [1]

Venous thromboembolism is a multifactorial disease affecting the vascular system of the body. It results in deep venous thrombosis and pulmonary embolism. The prior is characterized by the formation of a thrombus within deep and large veins, obstructing normal blood flow. Such thrombosis formation often occurs in the lower body. If the thrombus breaks off, flows up to the pulmonary artery and blocks blood flow to part of the lung, it is known as a pulmonary embolism. [1]

Peripheral arterial disease involves the development of plaque in the peripheral arteries, accordingly, causing interruption of blood flow to the limbs, particularly the legs. This may lead to pain and discomfort while walking, cramping in hips or calves and fatigue. [1]

1.1.1 Epidemiology of cardiovascular disease

Cardiovascular diseases are common, have a poor survival rate and have a rising prevalence globally. The number of cases almost doubled from 271 million in 1990 to 523 million in 2019. Further, the deaths caused by CVD increased from 12.1 million to 18.6 million over the same time period, with one third of the deaths in 2019 being in individuals between ages 30 and 70 [2]. Disability-adjusted life years (DALYs), which combine years of life lost due to premature death and disability caused by the condition [3], also increased worldwide and years lived with disability doubled from 17.7 million in 1990 to 34.4 million in 2019 [2]. CVD also presents a major economic burden both in direct costs and indirect costs, the prior being related to hospitalizations, drugs, physician visits etc., the latter being related to mortality and morbidity [4]. In 2003, the overall cost related to CVD in the EU was 169 billion euros. Of these costs, 62% percent were healthcare-related: 21% due to productivity loss and 17% due to informal costs [5].

1.1.2 Women and cardiovascular disease

Cardiovascular disease is commonly seen as a "man's problem", occurring in inactive men with an unhealthy lifestyle. Indeed, after age-standardizing, the rates of CVD are higher in men than in women. CVD, specifically coronary heart disease (CHD) and stroke, was the leading cause of both death and DALYs in men in 2017. However, the same year CHD and stroke were also the main killers of women worldwide [6]. Regarding DALYs in women, CVDs were amongst the most common causes globally, with CHD and stroke placing second and third respectively. In the US, CHD was the top leading cause of DALYs for women in 2017 [6]. Sex differences in DALYs also change depending on age. In 2019, the most extreme differences in total DALYs due to CVD were greater between ages 30 and 60, in which men had the highest, and after the age of 80, in which women had the highest [2].

Whilst many of the common risk factors for CVD such as smoking, high cholesterol etc. are shared between men and women, female-specific risk factors include adverse pregnancy outcomes and estrogen drop [7]. It was commonly believed and accepted that estrogen delays occurrence of CVD in fertile women, however epidemiological evidence shows that the rate of increase of CVD related to increasing age does not get any steeper around the age of when menopause would typically occur (between ages 45-55) [8]. Further, when post-menopausal women were treated with exogenous estrogen, no benefit related to CVD was found [9]. Other risk factors that are specific to women include those that are related to reproduction and include early menarche, early menopause, as well as a history of hysterectomy. Furthermore, amongst women who have given birth, early age of first birth, history of miscarriage and/or history of stillbirth, gestational diabetes mellitus, and preeclampsia are all correlated with an increased risk of CVD [6]. In fact, for preeclampsia, multiple studies show that in women with previous preeclampsia versus without the relative risk of developing CHD was 2.33, stroke 2.03, and overall mortality due to CVD was 2.29 [6].

The weakness, however, of studies involving pregnancy is that the maternal data used is usually obtained from registries lacking data on typical CVD risk factors in the mother both prior to and after pregnancy. Furthermore, such risk factors, including obesity, insulin resistance, hypertension and a family history of CVD, amongst others [10], are not only risk factors for developing CVD, but also for developing preeclampsia in and of itself. Therefore, it may be difficult to determine if preeclampsia is simply an unmasking of already existing cardiovascular conditions in the mother, rather than preeclampsia influencing future CVD risk directly [6]. As a result, common risk factors remain the common explanation for the link between the two conditions. However, even after correcting for these risk factors, preeclampsia still seems to confer an increased CVD risk [11, 12]. This suggests that the mother never fully recovers from the damage that occurs during preeclampsia; the consequence being a worsening of pre-existing risk factors or even induction of risk not already present [12].

Despite the obvious findings that CVD does not primarily affect men, women are still underrepresented in research related to these diseases. Since 2006, the mean percentage of female contribution in CVD clinical trials was only 30%, and only 50% of total trials gave results stratified by sex [7]. The lack of evidence regarding prevention, diagnosis and treatment in women compared to men likely lead to lower quality in both care and therapeutic options.

Additionally, targeting a woman's obstetric history is not included as a preventative measure for CVD.

1.1.3 The cardiovascular system

The cardiovascular system is responsible for delivering oxygen and nutrients to all the cells of the body, its components consisting of the heart and the blood vessels. The vessels can further be divided into arteries and veins, the former transporting blood from the heart and to the body, the latter performing the opposite task. Oxygenated blood is transported from the heart through the aorta, the main artery, which then undergoes progressive branching into the distal arteries, arterioles and, lastly, to the smallest arteries, known as the capillaries [13, 14]. The capillaries form the capillary bed, which is where exchange of nutrients and waste products between tissue and blood occur. The capillary bed exits into venules which further fuses with the veins, carrying deoxygenated blood back to the heart [15].

Arteries consist of three morphologically distinct layers: the intima, the media and the adventitia, referring to the inner, middle and outer layers respectively. As tissue is not solely made up of only cells, each layer consists of a different composition of cells and extracellular matrix components occupying the intercellular space. The components of the extracellular matrix are primarily proteoglycans and fibrous proteins. The function of the extracellular matrix varies from affecting cell development and behavior to providing strength and structure. Immediately after heart contraction, the blood pressure is high and thus there is a need for thick and elastic arterial walls to accommodate the accompanied stress [16]. A schematic representation of a healthy artery showing mainly the intima and media can be seen in figure 1.

The innermost layer of the arteries, the intima, is made up of the fibrous protein collagen and smooth muscle cells, bound by an elastic membrane against the media. On the luminal side, the intima is covered by a layer of endothelial cells, creating the necessary barrier to the arterial lumen. At birth the intima consists of a single endothelial monolayer, but continuously undergoes cell proliferation until maturity, after 25-30 years. This growth, termed diffuse intimal thickening, has been demonstrated in coronary, carotid and iliac arteries, to mention a few [17]. The endothelial layer regulates vascular tone, homeostasis and immune cell recruitment. The media is a highly organized layer consisting of contractile smooth muscle cells, elastin and collagen. Lastly, the adventitia is rich in collagen and has a sparce cellular population, thereby yielding support and shape. Due to their distance to the arterial lumen, the

adventitia and outer parts of the media are supplied with oxygen and nutrients through their own blood vessels termed the vasa vasorum, literally meaning vessel to vessel. Vasa vasorum can originate from the arterial lumen or arteries elsewhere. [13, 18-21]

1.2 The immune system

Fighting the ever-going battle against the foreign pathogens around us, the immune system comprises a complex network of a multitude of different, specialized cells and their molecular products. The immune system can, broadly speaking, be divided into two branches: the innate and the adaptive. The innate immune system provides a generalized non-specific first line of defense. This includes physical barriers such as the skin or the mucous layer covering the epithelial layer of the respiratory tract. Immune cells of the innate system include macrophages, dendritic cells and neutrophils, amongst others. At the molecular level, the innate system also holds soluble proteins, either as a residential feature, in for example mucous, or secreted by immune cells upon their activation. The latter includes chemokines which stimulate migration of inflammatory leukocytes. Molecules of the innate immune system also include receptors on cells which can detect certain molecular patterns only harbored by invading pathogens, known as pathogen-associated molecular patterns (PAMPs). The receptors that recognize these are typically located on immune cells but can also be found on non-immune cells such as epithelial cells or keratinocytes. Because the receptors are present on a multitude of cells, the response through this mechanism is rapid and thus represents the initial response against danger. [22] [23]

Different from the innate immune system, an essential feature of the adaptive immune system is specificity rather than general pattern recognition. The response is stimulated by specific receptors on T and B cells which recognize a specific antigen, i.e., any substance that can elicit an immune response. The receptors are named T cell receptors and B cell receptors, the latter commonly known as immunoglobulin (Ig) or antibodies. Based on complex rearrangements of the genes encoding these receptors and assembly of genetic elements, millions of different T and B cell receptors can be produced. [22, 23]

A main function of T cells is to destroy infected cells which have pathogenic material intracellularly including viral particles and intracellular bacteria. Therefore, the T cells must be able to detect both a self and a non-self-component simultaneously. This is solved through the

major histocompatibility complex, a cell surface molecule that can bind and present molecular compounds that are present within the cell. B cells are characterized by their Ig production in response to an antigen, which can be both membrane-bound and secreted out of the cell. The latter marks the antigen-bearing target for destruction through activating multiple cascade pathways. Upon antigen exposure, the B cell can respond in a T cell dependent or independent manner. The former occurs when the triggering antigen is also recognized by the T cells. In such cases, the T cells will facilitate B cell maturation, which includes increased antibody affinity for the antigen and is associated with immunological memory. Immunological memory is defined as a rapid immune response with high-affinity antibodies if exposed to the same antigen a second time. [22, 23]

The process of immune system activation in response to harmful events is known as inflammation, where the goal is removal of the inciting harmful event, resolution of the acute inflammation and restoration of tissue homeostasis. The general inflammatory pathway cascade can be summarized in a few steps; recognition of detrimental events by cell surface receptors, activation of inflammatory pathways, release of inflammatory markers and finally, the recruitment of inflammatory cells. The pattern recognized can be from pathogens (PAMPs), as briefly touched upon, or from damaged cells or tissue which are termed danger-associated molecular patterns, abbreviated DAMPs. The latter is therefore capable of activating an inflammatory response in the absence of pathogens [24].

1.3 Atherosclerosis

Arteriosclerosis refers to hardening of the arterial walls, derived from the Greek word's arteria, meaning artery, sclerosis, meaning hardening and finally the suffix -osis signifying an increase [25, 26]. The term is generic and can further be divided into three different disorders: atherosclerosis, arteriolosclerosis and Monckeberg medial calcific sclerosis (MMCS). The terms are often used interchangeably despite them describing different concepts. MMCS involves calcification of the internal elastic lamina or in the middle layer of the muscular arteries. Arteriolosclerosis describes hyaline or cellular thickening of the arterioles. Finally, atherosclerosis, although a word seemingly like "arteriosclerosis", the first portion of the term, athero, does not refer to arteries but rather the Greek word for porridge or gruel. This likely refers to the appearance of the fatty components found within atherosclerotic plaques. The latter portion, sclerosis, refers to stiffening and thus the word can be seen as meaning "stiffened by

fat". Indeed, it is characterized by the accumulation of lipids and fibrous elements within the arteries, as well as chronic inflammation [27]. Atherosclerosis is primarily a disease of the large arteries and can thus affect multiple areas of the body, including the heart and brain. Atherosclerosis remains the major cause for cardiovascular disease worldwide and will be the focus of the present work.

1.3.1 Initiation

It is widely accepted that low density lipoprotein (LDL), consisting of lipoprotein with a cholesterol center, plays a role in atherogenesis, and this has further been shown in genetic, epidemiologic and clinical studies in humans [28] and in animal experiments [29] [30]. It is important to note that LDL holds physiologically necessary levels of 0.5-1mM/L, the reference being new-born babies and mammals [31] [32], and that it is the excess that drives atherosclerosis development. For example, familial hypercholesterolemia is a disorder caused by a loss-of-function allele for the LDL receptor gene, a protein important for clearing LDL from the blood. The disorder is thus characterized by significantly elevated levels of blood cholesterol and along with-it early development of atherosclerosis. Individuals heterozygous for the condition often have circulating LDL of 4.5-12mM/L and will develop coronary heart disease before age 55-60, differing from their homozygous counterparts which will hold levels of 13-30mM/L and almost inevitably will develop atherosclerosis in childhood or early adolescence, most of them dying before the age of 20 if left untreated [33]. Moreover, perhaps the most compelling evidence for a causal relationship between LDL and atherogenesis lies in the therapeutics that target lowering LDL, which consequently show a risk reduction in developing cardiovascular disease [28]. Despite what seems to be clear causal evidence, the exact mechanism for how excess LDL causes atherosclerosis remains unclear.

The response-to-retention hypothesis states that subendothelial retention of LDL is the only absolute requirement for atherosclerosis development, regardless of the initiating factors that permit lipid infiltration. Under normal, healthy conditions, the endothelial layer is semi-permeable, thereby regulating cellular and molecular traffic between the blood and surrounding tissues. However, the endothelial layer may be disrupted or dysfunctional, leading to increased permeability. This is thought to allow the entrance of LDL into the intima. Examples of endothelium-disrupting agents are high blood pressure, increased blood-lipid and blood-glucose levels and smoking, all which are risk factors associated with cardiovascular disease.

Endothelial cells also undergo morphological changes in the branched areas of the arteries, due to non-uniform blood flow, making these areas preferred lesion sites. [7,9] [34, 35]. According to the "response-to-injury" hypothesis, lipid deposition and invasion of immune cells is initiated by damage to the endothelium [36]. Interestingly, endothelial progenitor cells (EPCs) may play a protective role against lesion development due to dysfunctional or damaged endothelium [37]. EPCs are bone marrow-derived precursor endothelial cells which migrate to the site of endothelial injury or dysfunction, where they may contribute to neovascularization and re-endothelialization [38, 39]. Studies suggest that low levels of circulating EPCs may facilitate CVD progression [40, 41].

Once in the intima, LDL may undergo modifications, an example being oxidization. The oxidized LDL exhibit pro-inflammatory properties, whereby they can activate the endothelial layer, subsequently leading to monocyte recruitment. The monocytes adhere to the endothelium and enter through to the intima, where they can further differentiate into macrophages. The macrophages bind to lipoproteins, and subsequently engulf the bound particle. Upon this event, the macrophages become lipid-filled *foam cells*. Of cellular components, the macrophages dominate in the intima, however T-cells also enter through the endothelial layer where they regulate functions of the innate immune system, endothelial- and smooth muscle cells. Furthermore, in response to the accumulation of leukocytes, smooth muscle cells undergo a phenotypical shift, in which they migrate to the intima from the media [42].

1.3.2 Inflammation: a link between risk factors and atherosclerosis

The concept of atherosclerosis has experienced a change over the years from being seen as a primarily lipid-laden disease to a process driven by both the innate and the adaptive immune system. The frequently mentioned macrophages make up the largest contribution of the innate immune system, however other innate immune cells such as dendritic and mast cells have also been found within atherosclerotic plaques [43]. Of the adaptive immune system, different subtypes of B and T cells are present in atherosclerosis in both mice and humans, thought to have both a pro- and anti-inflammatory effect. Of course, no immune response takes place without an initial trigger. In atherosclerosis, key antigens are thought to be oxidized-LDL, LDL and ApoB, the core protein of LDL [44].

Inflammation can be seen as the link between the classical risk factors and atherosclerosis; hypertension, tobacco use and insulin resistance. The exact mechanism by which they

contribute to atherogenesis is unclear, however what is known is that these risk factors are involved in activating inflammation, which in turn may influence the cells in the artery wall and thereby drive atherosclerosis. For example, angiotension II, a peptide which plays a large role in hypertension by constricting the blood vessels, activates NF-kappaB in monocytes. NF-kappaB is a transcription factor that participates in most inflammatory signaling pathways, and monocytes are a main contributor in atherosclerosis [45]. Studies have also shown an activation of T-cells by angiotensin II, which also is connected to atherogenesis [46]. In the case of tobacco use, it has been shown that smoke-components interact with circulating cytokines to up-regulate the expression of inflammatory genes in vascular endothelial cells, thereby contributing to endothelial dysfunction [47]. Finally, it has been shown that a therapeutic drug targeting the interleukin-1beta, a pro-inflammatory cytokine, significantly lowers the risk of cardiovascular events without affecting the lipid composition [48].

Despite inflammation being vital to health, it can have a detrimental effect if the response is prolonged or too strong. Resolution of inflammation and return to homeostasis is therefore a highly important and highly regulated process. The event of atherosclerosis can be deemed a matter where such a mechanism is defective and can be said to be a disease characterized by chronic, non-resolving, low-grade inflammation. [2, 49]

1.3.3 Progression

Once the initiation of atherosclerosis is established, the plaque progresses through continued lipid accumulation and the increase of foam cells that follows. These foam cells can be a result of lipid-engorged macrophages or smooth muscle cells, the former being more widely recognized. Further, the foam cells may undergo programmed cell death, though the exact reason why is unknown [50]. This results in leakage of all their lipid-filled contents into the plaque, along with the remains of the dead cells. The intima does harbor resident smooth muscle cells, although the main contribution to the plaque is proliferation and migration from the media [42]. Once in the intima, due to their phenotypical shift smooth muscle cells can produce and secrete extracellular components which make up most of the stereotypical bulk seen in advanced atherosclerosis. Calcification may also occur in such instances through apoptotic foam cell debris and macrophage-released cytokines. The cytokines in turn stimulate osteogenic, bone synthesis, differentiation and mineralization of smooth muscle cells. The process of calcification seen in atherosclerosis is like that seen in bone formation [51].

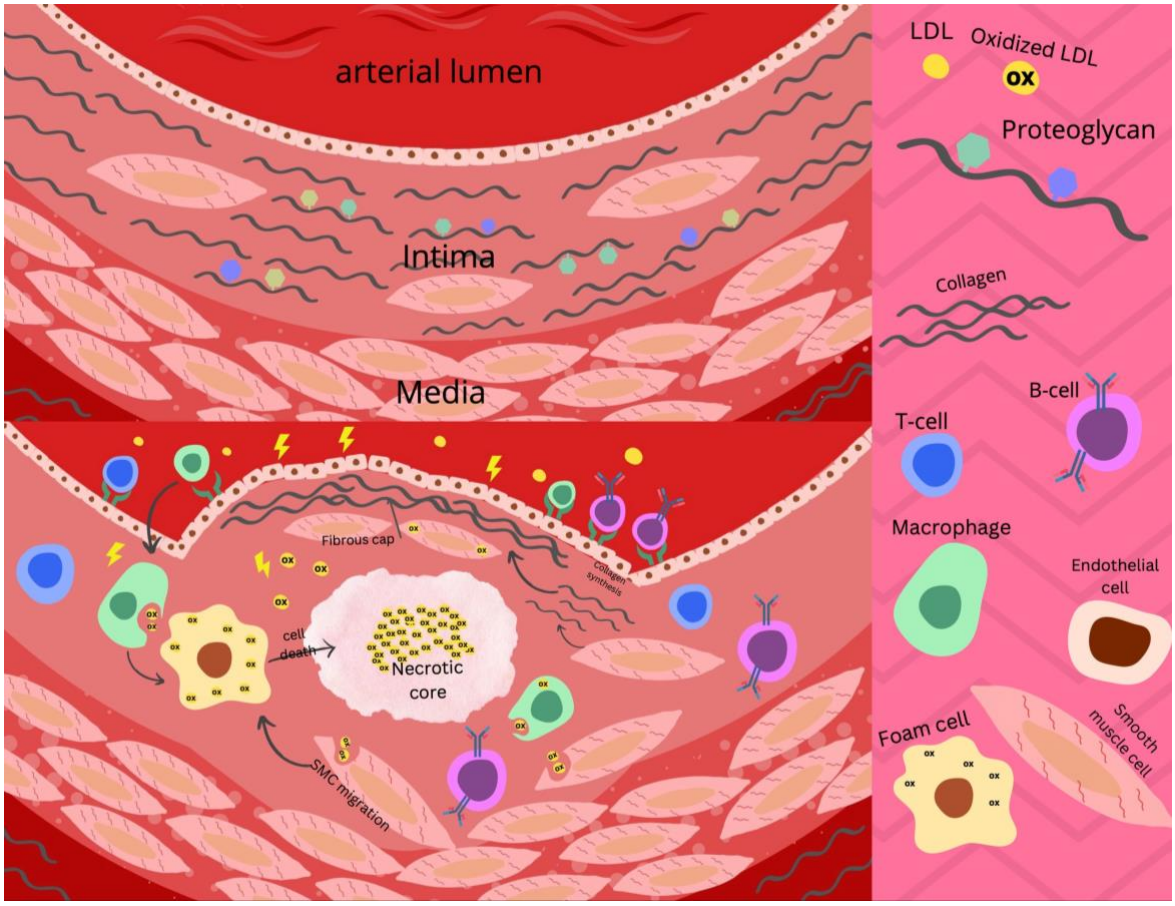

Figure 1. *Schematic representation of healthy artery wall (top) and atherogenesis according to the response-to-injury hypothesis (bottom). Dysfunctional endothelium permits entry of LDL into the intima whereby it becomes oxidized. This leads to monocyte recruitment to the endothelium and entry to the intima where they differentiate to macrophages. Macrophages and smooth muscle cells engulf Ox-LDL, thereby becoming foam cells which undergo apoptosis, their contents and remnants contributing to the necrotic core. Smooth muscle cells migrate from the media and produce collagen, creating the fibrous cap overlying the core. T cells and B cells are also found within atherosclerotic lesions.*

1.3.4 Complications

During much of the atherogenesis timeline, the plaque bulges away from the lumen and thereby preserves the free flow of the arterial lumen. However, as the plaque progresses further, remodeling of the arterial wall may occur through extracellular matrix degradation. Consequently, this allows for increased luminal projection of the plaque and may lead to restricted flow through the lumen, which can give ischemia and symptoms of angina. [52] [53]

Moreover, the extracellular matrix components may form a fibrous cap over the lipid core of the plaque. This cap, in turn, can rupture as a result of impaired collagen production by smooth muscle cells and released collagenases from inflammatory cells. The former results in lacking maintenance of the cap skeleton, the latter results in active breakdown of structural cap components. Either way, a fissured fibrous cap can leak the contents of the atherosclerotic lesion into the arterial lumen. A sequence of coagulation events then leads to the formation of a platelet-rich thrombus [42].

Among the constituents of the atherosclerotic plaque exposed to the lumen upon plaque rupture is the tissue factor protein that is the initial triggering event. Its exposure to the blood activates platelet recruitment and the following coagulation cascade. Both involve activation of inactive circulatory components of the blood, the main goal being to halt the bleeding. Platelets are small cells lacking a nucleus, however still capable of protein expression through their harboring RNA. Their production occurs mainly in the bone marrow, after which they circulate in the blood for 7-10 days before being eliminated in the liver or spleen [54]. If damage, dysfunction or rupture of the endothelium occurs, platelets are recruited and subsequently adhere to the site of injury. Their secure and stable binding is primarily facilitated by platelet receptors of the intimal collagen [35]. Further, tissue factor protein of the intima will interact with plasma factor of the blood, whereby a complex capable of converting prothrombin to thrombin is formed. The thrombin may then cleave fibrinogen to fibrin and promote cross-linking between the fibrins, thereby promoting stabilization. [55] [54]

In the early stages of thrombosis, it can cause symptoms of angina or small parts may break away, traveling to a smaller vessel elsewhere where it may have an occluding effect. However, the real dreaded consequence of atherosclerosis is thrombus progression and growth, which can lead to complete occlusion of the lumen and thereby cause ischemic events such as a stroke and myocardial infarction. [54]

Thrombosis is often associated with a following serious ischemic event; however, it may become organized within the plaque, thereby contributing to the progression of atherosclerosis. This hypothesis is particularly supported by findings showing that some plaques progress in a very rapid manner, which differs from the linear progression if it were simply an accumulation of lipids, and lipid-engorged cells, over time. Instead, it has been suggested, based on monitoring plaques at different time intervals, that it is a highly unpredictable process,

progressing in a non-linear manner [56]. This hypothesis is also supported by the findings of thrombus components within atherosclerotic plaques and vice versa [57].

The susceptibility of a plaque rupturing may vary. They have traditionally been categorized, in a simplified manner, into two: vulnerable and more stable plaques. It has been shown that plaques that rupture have possessed a large lipid-core and a thin fibrous cap [58]. It has also been shown that rabbits fed on a low-fat diet, which lowers the risk of cardiovascular events, developed plaques exhibiting less lipid content and fibrous plaque richer in collagen, thus implying increased stability [59].

1.4 Pregnancy

In pregnancy, an embryo will grow and develop inside the uterus of a woman. The uterus is a hollow organ located in the lower abdomen of all females, involved in menstruation, gestation, delivery and birth. It consists of three different tissue layers. The endometrial inner cavity lining, the muscular myometrium layer and the outer perimetrium. The uterus is mainly supplied by the uterine arteries, branching into arcuate arteries in the myometrium and further into basal and spiral arteries in the endometrium. The spiral arteries continue to the endometrial surface and are so-called because of their coiled appearance [6, 60] [61] (figure 2, left). In the very early days of pregnancy, the growing embryo consists of a multi-cellular ball-shaped blastocyst. The blastocyst consists of two cell types: the inner cell mass and the surrounding trophectoderm. The former will give rise to the fetus and some extraembryonic structures, whilst the latter will give rise to the umbilical cord and the placenta [11]. Therefore, although not part of the growing embryo itself, the placenta is an organ fully of fetal descent.

The placenta at term can be described as a discoid organ, with a diameter average of about 22cm and a central average thickness of 2.5cm. Its two surfaces consist of the chorion plate which faces towards the growing fetus, to which also the umbilical cord is attached, and the basal plate, which meets the maternal endometrium, termed decidua basalis during pregnancy. Between the placenta and the decidua basalis lays a cavity called the intervillous space which holds 30 to 40 extensively branched villous trees, each derived from a stem villous of the chorion. Emerging through the decidua basalis are spiral arteries, bathing the intervillous space and the fetal villous lobes with blood. Each lobe is covered by a multinucleated layer called the syncytium with no openings towards the intervillous space [62] (figure 2, middle). Each villus

is vascularized through the fetal capillary network and thus, can be thought of as a single, independent unit of exchange between mother and fetus. This includes transport of gases, glucose and amino acids and more [63]. Oxygenated blood then exits through the uterine veins [62].

Aside from nutrient exchange, the placenta also anchors itself to the uterine wall, where it secretes hormones with profound effects on maternal metabolism and the maternal immune system [62]. The diversity of function lays in the differentiation and proliferation of what is called trophoblastic stem cells [64]. A descendant of such cells are the cytotrophoblasts, which can further be divided into two; villous cytotrophoblasts (VT) and extra-villous cytotrophoblasts (EVT). The villous cytotrophoblasts underlay the syncytial layer of the chorionic villi whilst the EVTs are responsible for invading maternal tissue. The EVT's take on \ tumor-like behavior, invading the uterine wall and migrating until the maternal spiral arteries are found. Initially, they will invade and plug the spiral arteries, causing a hypoxic environment which is necessary for early development of the placenta. In this period, the fetus is provided with nutrients through fluids secreted from endometrial glands [65].

The EVT invasion hits a peak at around 9-12 weeks of gestation, whereupon remodeling occurs [66] in which the cytotrophoblasts replace the endothelial and smooth muscle cells of the spiral arteries [67]. Upon this event, the initial hemodynamic character of the spiral arteries changes from high resistance to low resistance through the loss of the contractile smooth muscle. This remodeling ensures steady and low-velocity blood perfusion, submerging the chorionic villi in blood in the intervillous space [68, 69].[68, 69]. The reduced velocity is necessary for protection of the delicate chorionic villi of the placenta and to ensure enough time for transport of nutrients and oxygen. In healthy pregnancies, the depth of spiral artery remodeling reaches about one third of the myometrium [70].

1.4.1 Preeclampsia

Preeclampsia is a multisystem disorder that occurs during pregnancy which includes the de novo onset of hypertension accompanied by de novo proteinuria or other signs of de novo maternal organ dysfunction after 20 weeks' gestation [71]. It is a major contributor to mortality and morbidity in both neonates and mothers and each year is responsible for 50 000-60 000 deaths worldwide [72]. It is a syndrome rather than a single well-defined disease and presents itself in several forms, broadly categorized into an early-onset and late-onset form. The early-

onset form is defined by premature delivery before 34 weeks of gestation and is additionally associated with a higher incidence of fetal growth restriction (FGR). The late-onset form of preeclampsia involves delivery after 34 weeks and is not usually accompanied by FGR, however, the life-threatening complications eclampsia and HELLP (hemolysis, elevated liver enzymes, and low platelets) more commonly arise in the late-onset form [73]. The pathogenesis of preeclampsia remains unclear; however, a two-step model was proposed in 1991 which changed the concept of preeclampsia from being a maternal syndrome to a syndrome of the placenta [73, 74]. As mentioned above, remodeling occurs throughout the myometrium under normal conditions, however in preeclampsia it is usually confined the decidual layer, which, in turn, causes abnormal perfusion of the placenta (figure 2, right). The lack of spiral artery remodeling is thought to occur due to low EVT motility or to a hyperactive maternal immune system. According to the model, stage 1 of preeclampsia encompasses a pre-clinical stage characterized by poor placentation due to insufficient remodeling of the uterine arteries, thereby leading to abnormal perfusion of the placenta causing placental stress. This is then thought, in turn, to give the clinical picture seen in the mother: stage 2 [74].

Previously it has been thought that insufficient spiral artery remodeling would cause under-perfusion, resulting in a chronic low-oxygen state within the intervillous space. However, in 2009 it was shown that it is not the volume of blood flow that is affected but rather the motion of flow [75]. The lack of remodeling of the spiral arteries causes the inflow of blood to have a higher force as well as being more pulsatile, thereby damaging the chorionic villi. This in turn will cause release of placental factors, which in susceptible individuals will lead to the clinical picture seen in the mother [75]. The understanding of the connection between stage one and two of preeclampsia was greatly enhanced by findings that an imbalance between certain trophoblast markers was present in preeclampsia compared to normal pregnancies. The imbalance involved increased levels of the anti-angiogenic factors soluble Fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng), and decreased levels of the angiogenic factor placental growth factor (PlGF) [76] [77]. The evidence for an imbalance of these factors causing symptoms of preeclampsia is supported through animal studies. In rats with an excess of sFlt-1 signs like those of preeclampsia were observed [76]. Additionally, mice with decreased levels of the vessel dilator adrenomedullin, which is also found to be decreased in women with preeclampsia, induced similar symptoms. [69]

Many modifications and refinements have been introduced since the proposal of the original two-step model to explain incompatibilities between preeclampsia, risk factors and new findings. One such refinement model, described in 2014 [78], provided an explanation for late-onset preeclampsia without FGR, incompatible with insufficient placentation, which was lacking in the original two-stage model. The model from 2014 suggests a second, additional route to placental dysfunction occurring later in pregnancy, whereby it occurs due to the placenta outgrowing its uterine capacity. This leads to a compressed intervillous space, disturbing perfusion and causing stress to the syncytium. This can explain why late-onset preeclampsia is not commonly associated with FGR as the pregnancy has had a longer time period with a healthy placenta. An improved version of the model from 2019 also incorporates maternal risk factors, which include obesity, diabetes, chronic hypertension and autoimmune disease [73]. It is suggested that these may affect multiple pathways in preeclampsia, including initial placentation, placental function and lastly, placental size. Thus, risk factors associated with inflammation can contribute to preeclampsia through propagating placental dysfunction and the shedding of placental factors. Additionally, because women with these risk factors have a higher level of generalized inflammation prior to pregnancy, they more rapidly reach a severe level of inflammation in response to the released placental factors.

During any pregnancy, debris from the syncytial layer of the chorionic villi is released into the intervillous space of the placenta. This includes nucleated syncytial material termed syncytial nuclear aggregates, subcellular and molecular particles, and cell-free fetal DNA. However, in preeclamptic patients the level of syncytial material in the blood is significantly increased compared to healthy pregnancies. Moreover, these microparticles have been shown to be involved in endothelial dysfunction and stimulation of cells of the innate immune system. Together, this may imply that these particles can in part be what causes the maternal stress in preeclampsia.[79]

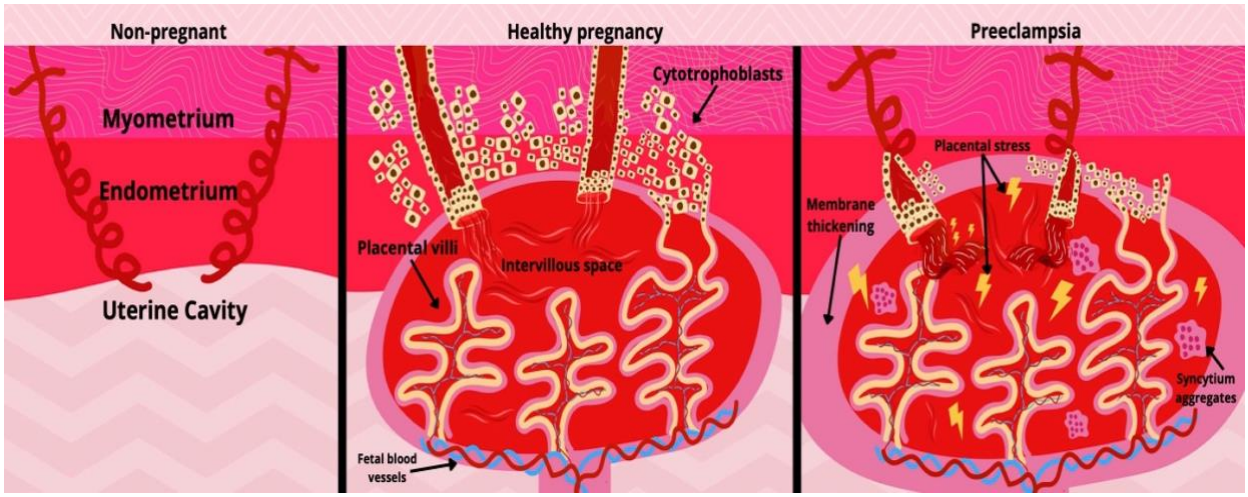

***Figure 2**. Schematic representation of the uterine wall in a non-pregnant woman (to the left), placentation in a healthy pregnancy (middle) and placentation in early-onset preeclampsia (to the right). In healthy pregnancies the cytotrophoblasts will invade maternal tissue and remodel the uterine spiral arteries, allowing for a steady, calm flow into the intervillous space (IVS). In early onset preeclamptic pregnancies, insufficient cytotrophoblast invasion leads to shallow remodeling, causing a pulsatile flow of blood into the IVS, causing oxidative stress and release of placental factors. Increased placental membrane thickening is also a morphological change seen in* the *placentas of preeclampsia [80]. Maternal veins are not included in this representation.*

1.5 Fetal microchimerism

A bidirectional trafficking of cells and cell-free DNA between mother and fetus occurs during pregnancy, thereby resulting in the mother and fetus possessing genetic material from one another during pregnancy, and even after delivery. The exchange has been shown to be asymmetric, with more genetic material both in the form cells and cell-free DNA being transferred from mother to fetus than vice versa [81]. The clearance-kinetics differ between cell-free DNA and transferred cells. Cell-free DNA is rapidly removed from mothers' circulation following delivery, contrasting from cells of fetal origin, which can persist for several decades after birth [82, 83]. The presence and persistence of fetal cells in maternal circulation is named fetal microchimerism, abbreviated FMC accordingly. The term microchimerism can be defined as a small group of cells from one individual, existing within another, genetically distinct, individual. Looking into the etymology of the term, the word chimera refers to a monstrous hybrid-creature of Greek mythology. The chimera is usually depicted as a lion with a goat's head emerging from its back and the lion's tail ending in the head of a snake. Micro is a prefix for describing something small, presumably referring to the

small amounts of semi- or fully allogeneic cells in microchimerism, as one of the genetically distinct entities is present in a much smaller scale than the other. [84] [85]

Fetal cells in maternal circulation were first documented by Schmorl in 1893. He detected multinucleated cells in the blood vessels of the lungs of women who had died of eclampsia, a severe form of pre-eclampsia, and concluded that they must be of either placental or decidual origin. Furthermore, he also suggested that the underlying cause of this fetal cell invasion of the maternal body was a dysfunctional placenta [86]. Lastly, FMC is thought to occur within all placental mammals, found in cows, mice and primates, amongst others [87].

Regarding the onset of FMC, a study looking at the quantity of fetal cells throughout pregnancy found fetal cells in maternal circulation as early as gestational week 4 in some women, however by parturition fetal cells were present in all the women included in the study. A correlation between the concentration of fetal cells and number of weeks of gestation was also found, with a clear peak at parturition. In addition, FMC has been shown to be present both more frequently and in higher concentrations in the peripheral blood of women with preeclampsia compared to the gestational age matched healthy control group [88]. Another study looking at FMC postpartum reported that both the concentration of fetal cells and the overall detection among the test subjects faced a rapid decline, although some women were still found to harbor fetal-origin cells in their circulation about 1 month after delivery [89].

1.5.1 Maternal locations of FMC

As briefly touched upon, fetal cells can be found in maternal circulation up to 27 years postpartum. These cells were found to express both CD38 and CD34, markers for lymphoid progenitor cells thereby having the ability to differentiate into mature immune cells [24]. Additionally, fetal microchimeric cells with markers for T cells, B cells, monocytes and dendritic cells have been found in the blood of some women up to 38 years after delivery [90]. Cells of fetal origin have also been found in multiple regions of the brain, both from women without any evidence of neurological disease and in those suffering from Alzheimer's [91]. Findings also suggest that FMC is present in virally diseased maternal tissue, including hepatitis C, a liver disease caused by a viral infection, and cervical cancer which is associated with human papilloma virus [92] [93]. Based on these findings, it was hypothesized that fetal cells were involved in the maternal response to injury. A study was thereby conducted aiming to identify different markers expressed by microchimeric fetal cells [94], revealing their cellular

identity. Fetal cells from diseased maternal tissue, including intestine, spleen, lymph nodes and gallbladder, were detected and their markers investigated. Indeed, a proportion of cells from each tissue type were found to bear epithelial, leukocyte and hepatocyte markers, thus providing evidence that fetal-origin cells can differentiate into various phenotypes within maternal tissues [94].

1.5.2 FMC as an inflammatory agent

Systemic sclerosis is an autoimmune disease in which the body, mistakenly, attacks its own tissues, thereby leading to increased fibrosis i.e., scar tissue. When looking at a selection of tissues from women suffering from systemic sclerosis, male cells were found in at least one tissue type of each woman. As all the women had given birth to sons it was concluded that the cells were likely of fetal origin. However, one of the women had previously received a blood transfusion and thus could not be excluded as a possible source. Contrastingly, in the control group consisting of healthy women who had died of causes unrelated to autoimmune disease, no male cells were detected in the same tissues [95]. Furthermore, in the circulation of women with systemic sclerosis, fetal cells were found in almost half the women, in contrast to the healthy control group, which held a frequency of only 4% [96]. Similar patterns are seen in other autoimmune diseases such as the thyroid-affecting Grave's disease and Hashimoto's, in which a larger proportion of women were found to harbor chimeric cells in their thyroid glands as opposed to the control group [97] [98]. In the inflammatory skin condition polymorphic eruptions of pregnancy, which occurs during pregnancy, fetal cells were found in the skin lesions in 60% of women with the disease contrasting from skin samples of the healthy control group, in which no fetal cells were detected [99]. However, the findings of fetal microchimerism in autoimmune disease and other inflammatory conditions are contradicting, with some studies finding increased FMC in autoimmune diseased tissue and others finding no such association [100] [101].

Caesarean section is a common surgical procedure that is performed to deliver the baby and is an alternative when traditional birth through the vaginal canal is not possible or safe. The healing process that follows the incision in the maternal abdomen, and injuries in general, consists of three steps: inflammation, tissue formation and tissue remodeling. The wound will almost reach full strength after 3 months; however, the skin will never regain the properties it once had prior to injury [102]. This differs to fetal embryo healing, in which normal skin with

no scarring is accomplished post injury [103]. The differences in the two processes lay in extracellular matrix components, the inflammatory response to tissue injury and cytokine and growth factor profiles amongst others [104]. Thus, it was suggested that perhaps these differences would result in a maternal wound-healing process more like that of the fetus, following delivery. This change would be due to fetal microchimeric cells possessing a regenerative character. Furthermore, male cells, again presumed to be fetal in origin, were found in the epidermis of a healed caesarian scar. They held an epithelial-like morphology and were positive for cytokeratin, meaning they had differentiated into local skin cells [104]. Additionally, they were also found to possess markers of wound healing, such as TG1-beta1 and TG1-beta3, possibly confirming their involvement in the healing process. Because various studies have shown that cells derived from the bone marrow contribute to the regeneration and repair of skin, the thought is that not only maternal cells are recruited from the bone marrow to the site of injury, but also fetal cells residing in the bone marrow. The latter is supported by a finding made in 2004, in which bone marrow derived mesenchymal cells of presumed fetal origin were identified. It was thereby suggested that fetal cells transferred to maternal blood engraft in the bone marrow and possibly remain there for life [105]. Considering the fetal cells in the C-section scar, it was also hypothesized that fetal epithelial stem cells engraft into maternal skin during pregnancy, whereupon they later can differentiate and proliferate into mature keratinocytes [104].

In a possibly similar matter, was the finding of increased FMC in both the circulation and the heart of female mice following induction of a myocardial infarction (MI) [106]. The cells were found to persist for two weeks following the MI, mainly surrounding the injured tissue. Further, after three weeks the cells were found differentiated into mature cardiac cells, including smooth muscle cells, endothelial cells, and cardiomyocytes. The latter being responsible for the contractile force of the heart. Thus, this supports that fetal cells play a role in the response to maternal injury [106].

1.5.3 FMC and expanded immune tolerance

Pregnancy introduces what can be seen as a biological enigma. Women with a fully functional, healthy immune system, should reject foreign material invading the body through it being recognized as non-self. However, a fetus, which is partly genetically different to the mother, i.e., semi-allogeneic, is carried for nine months without immune complications. Furthermore,

the autoimmune diseases rheumatoid arthritis and multiple sclerosis show temporary improvement during pregnancy, providing evidence for modifications of the maternal immune system [107, 108]. However, the improvement is dependent on a class of genes called HLA: human leukocyte antigens. These encode proteins important to the human immune system, necessary for recognizing, binding and presenting antigens of foreign particles. The genes are highly polymorphic, i.e., there are a multitude of different alleles, gene variants, among the population [109] [110]. Every individual will inherit one HLA-allele from their mother and father respectively. Regarding rheumatoid arthritis, a greater dissimilarity between the maternal and fetal non-shared HLA-alleles, shows greater improvement of the disease during pregnancy [111].

The regulation occurring during pregnancy may be facilitated by the maternal exposure to fetal cells within maternal circulation. T cells, of the T regulatory CD4+ type (Treg), have been shown to have a silencing effect on other immune components, thereby sparking an interest in its effect on pregnancy [112]. Furthermore, one would expect these fetal microchimeric cells to interact with T cells of the maternal immune system. Upon Treg differentiation, Tregs are exposed to self-antigens and thus acquire surface proteins specific to the self, thereby able to suppress autoimmune reactions if they were to occur. Further, during pregnancy an expansion of antigens to which the mother is exposed may take place thereby expanding the Treg repertoire. Such an expansion can be measured through targeting the expression of certain cellular markers within a T cell population and the detection of expanded Tregs are found to increase in pregnancy compared to non-pregnant controls [112]. A similar mechanism is thought to be present in B-cells [113].

2 Aims

My hypothesis states that fetal microchimerism (FMC), obtained during pregnancy and sequestered in the maternal bone marrow, may be recruited as immune cells or endothelial progenitor cells to sites of endothelial dysfunction where their semi-allogeneic nature promotes inflammation. This, in turn, may contribute to atherosclerosis and clinical cardiovascular disease (CVD). Increased fetal cell-trafficking is seen in patients with preeclampsia and may play a role in the epidemiologically well-documented increased CVD risk of women with this condition. Moreover, preeclampsia may be an indication of poor tolerance to the semi-allogeneic fetus during pregnancy. Incorporation of such immune-incompatible cells into the vessel walls may promote inflammation to a greater extent than FMC derived from pregnancies with higher maternal-fetal immune tolerance. My goal is to establish methods that enable for detection of fetal microchimerism from female tissue. In this project, the aim is to:

1. Establish methods for detecting the presence and quantity of FMC in maternal tissues using droplet digital PCR.

2. Establish methods for spatial detection of FMC in maternal tissues using fluorescence in situ hybridization.

3. Investigate the presence of FMC in atherosclerotic carotid endarterectomy biopsies from postmenopausal women.

3 Methods

3.1 Tissue collection

3.1.1 Atherosclerotic plaque

Atherosclerotic plaque tissue was collected from the Carotid Biobank. The tissues fixed in formalin were collected in the years 2015-2019. Atherosclerotic tissue is removed from patients through carotid endarterectomy, where an incision is made along the neck to access the carotid artery, the artery opened, and the atherosclerotic plaque removed. The main purpose of the surgery is to reduce the patient's risk of stroke or cerebrovascular disease [114]. The atherosclerotic plaque tissue was a) stored at -80 °C for isolation of DNA or b) fixed in a formaldehyde solution, also called formalin, for imaging analysis. Formaldehyde interacts with macromolecules, including proteins and nucleic acids and creates covalent bonds between those that are adjacent in space [115]. For further processing of the formaldehyde fixed tissue, the plaque tissue from each patient was cut into four separate parts: two distal and two central.

The parts were thereafter dehydrated and embedded in paraffin in an embedding mold (formalin-fixed paraffin-embedded (FFPE)) at Dept. of Pathology, Oslo University Hospital. The Solid paraffin wax consists of a mixture of long hydrocarbons derived from petroleum, it is colorless and odorless [116]. At Dept. of Pathology the FFPE tissue was sectionized with a microtome, a cutting tool for preparing extremely thin slices. The sections were cut to 3 micrometers, about the same thickness as a cell, and placed on a glass slide. In this project, 15 frozen female samples and 1 male sample were used for DNA isolation and digital droplet PCR (ddPCR), while FFPE samples from 4 women and 1 man were used for fluorescence in situ hybridization (FISH) analysis.

The study was approved by the regional committee for Medical and Health Research Ethics in South-Eastern Norway (approval numbers: 2014/2078; 2009/5237; 2019/103, REK amendment 8949 COMPASS) and conducted in accordance with the principles of the Helsinki Declaration.

3.1.2 Placenta and decidua basalis

For validating FISH as a method for detecting male fetal cells it was performed on placental and decidua basalis tissue prior to being performed on atherosclerotic plaque. These tissues were collected from the Oslo Pregnancy Biobank containing tissue samples from women who have given birth at Oslo University Hospital from 2000 to 2018 [117]. Decidua basalis was collected through a vacuum suction technique in which vacuum suction is applied to the uterine wall after removal of the placenta during a caesarian section.

3.2 DNA isolation

The goal of DNA isolation is to obtain a homogenous DNA preparation whilst at the same time conserving the chemical and physical properties of the important genomic molecules in our case, to be used for PCR-detection of FMC. There are several different methods for isolating DNA, however, general steps include cell breakage, removal of protein and RNA, concentrating the DNA and finally, quantification and purity determination [118].

The primary step is to disrupt the tissue to facilitate cell lysis. Frozen, fresh atherosclerotic tissue was placed in a pre-cooled mortar, where it was grinded into fine, powdered tissue. A scalpel blade, frequently immersed in liquid nitrogen for maintenance of the cool temperature, was used to transfer the attained powdered frozen tissue into 1.5 ml tubes which was stored in a -80 °C freezer until cell lysis was to be performed. To avoid DNA-contamination during this step, all nearby surfaces and tools were thoroughly washed with Virkon, a disinfectant, and Ethanol. Further, cross-contamination from previous samples and between samples was avoided by defrosting the metal mortar, and all associated tools, followed by washing with Virkon and finally rinsing with filtered water. The metal mortar was then cooled down again by being put on dry ice (frozen carbon dioxide).

A DNA isolation kit from Qiagen (Qiagen, Germany) was used for cell lysis and further DNA extraction (DNeasy Blood & Tissue Kit). Tissue samples were first subjected to a tissue homogenizer. Contamination of tissue by the tissue homogenizer was avoided by bathing the wisp-part, ie. the part in direct contact with the tissue, in Virkon for approximately 5 minutes followed by rinsing with water and dried with a paper towel. 40 µl proteinase K and 360 µl buffer ATL was then added to the tubes containing the powdered tissue. The tubes were then vortexed and left to incubate at 57 °C for 1 hour in a dry water bath incubator.

Following tissue lysis, the tubes were vortexed for 15 seconds before adding 400 µl buffer AL and 400 µl ethanol (doubled amount). Lastly, the DNA was physically separated from the rest of the lysis solution containing protein and cell debris through a method called solid phase extraction, in which the DNA is bound to a silica membrane to facilitate purification. The mixture from each of the 16 tubes was pipetted into a DNeasy Mini spin column placed in a 2ml collection tube and centrifuged at 6000 x g for 1 minute, the collection tubes containing the flow-through were then discarded. After placing the spin columns in new 2 ml collection tubes, 500 µl buffer AW1 was added and thereafter centrifuged at 6000 x g for 1 minute. The collection tubes with flow-through were discarded. The spin columns were then transferred to new 2 ml collection tubes, 500 µl buffer AW2 added and centrifuging at 20 000 x g for 3 minutes. Each of the spin columns were then removed carefully from the collection tubes to avoid contamination of the spin column membrane with ethanol. The spin columns were then placed into clean 2 ml microcentrifuge tubes before adding 200 µl buffer AE directly onto the membrane of the spin column. The tubes were then left to incubate for 1 minute before being centrifuged at 6000 x g to elute the DNA. For maximum DNA yield, the elution step was repeated, as suggested in the protocol. The same microcentrifuge tube was used.

3.3 Digital droplet PCR (ddPCR)

3.3.1 Making the droplet digital PCR sample

The DNA isolated from the tissue was used to make the ddPCR reaction sample using the ddPCR Supermix for probes (No dUTP) from Bio-rad (Bio-Rad Laboratories, USA). Reaction mix, target primers/probes and DNase/RNase-free water were all mixed together according to the Bio-Rad protocol. The probes used in this experiment were the SRY probe with FAM fluorescence and the AG01 probe with HEX fluorescence. SRY targeted the Y chromosome for fetal microchimerism detection, and the AG01 targeted chromosome 1 as a control. The total reaction volume was 20 µl, with 10 of those being the supermix and 2 being the primers/probes. The amount of water and DNA sample varied depending on the original DNA concentration. The initial DNA concentration of each sample was measured using Qubit (Thermo Fisher Scientific Inc, USA) and the desired DNA input amount was set to 20ng. A full table of the different volumes used, and the initial concentrations of each sample, can be seen in table 1. The control sample was made beforehand, representing DNA coming from a woman with no

elder brothers nor sons and successfully showing no hits for the Y chromosome in a previous ddPCR run.

Table 1. Showing the different components used in the ddPCR reaction mix.

Sample	Sample DNA conc., ng/µl	Desired DNA input of reaction samples, ng	Volume of sample added to reaction mix, µl	Volume of water added to reaction mix, µl	Probes/Primers /Supermix Volume added to reaction mix, µl	Total volume of reaction mix, µl
1	10.8	20	1.85	6.15	12	20
2	2.86	20	6.993	1.007	12	20
3	5.70	20	3.509	4.491	12	20
4	11.6	20	1.724	6.276	12	20
5	4.98	20	4.016	3.984	12	20
6	14.0	20	1.429	6.571	12	20
7	23.6	20	0.847	7.153	12	20
8	13.8	20	1.449	6.551	12	20
9	20.8	20	0.961	7.039	12	20
10	13.8	20	1.449	6.551	12	20
11	2.73	20	7.326	0.674	12	20
12	10.4	20	1.923	6.007	12	20
13	3.43	20	5.830	2.17	12	20
14	8.08	20	2.475	5.525	12	20
15	4.32	20	4.629	3.371	12	20
16	8.52	20	2.347	5.653	12	20
Control	61.14	20	0.328	7.672	12	20

For the reaction mix, a set input amount of 20ng DNA was decided, whereby the needed volume from the DNA samples was calculated using their initial concentration. 10µl of the supermix and 2µl of the primers/probes were added for each reaction mix. Finally, variable amounts of water were added to achieve the final volume of 20 µl.

3.3.2 Droplet generation

Each sample was partitioned into as much as 20 thousand small droplets, each functioning as a micro reaction unit. This was achieved using the QX200 droplet generator from Bio-Rad. Firstly, the Bio-rad 8x3 cartridge was placed into its appropriate holder whereupon the reaction mix, and oil were pipetted into their appropriate wells. To avoid air bubbles, which can disrupt ideal droplet formation, the pipetting technique for the sample described in the protocol was followed carefully. This involved using 20 µl filtered tips, sliding them down the wall of the well to the bottom with an angle of 15 degrees. With the pipette tips at the bottom edge of the well, half of the sample was slowly dispensed. Thereafter, whilst dispensing the rest of the sample, the tip was drawn slowly up the wall without pushing the pipette plunger fully down. For this step, an 8-channel multi-pipette was used for convenience, transferring 20 µl of each reaction sample into each well of the droplet generator cartridge. 70 µl of the droplet generation oil was transferred in a normal manner into each of the oil wells in the cartridge. The gasket was then hooked over the cartridge and, in its holder, was placed in the QX200 droplet generator. The droplets were created through water-oil emulsion technology (figure 3) whereby each droplet contains at most a few target-DNA sequences, and all the necessary components for PCR [119]. After 2 minutes, the cartridge in its holder was taken out and the gasket discarded.

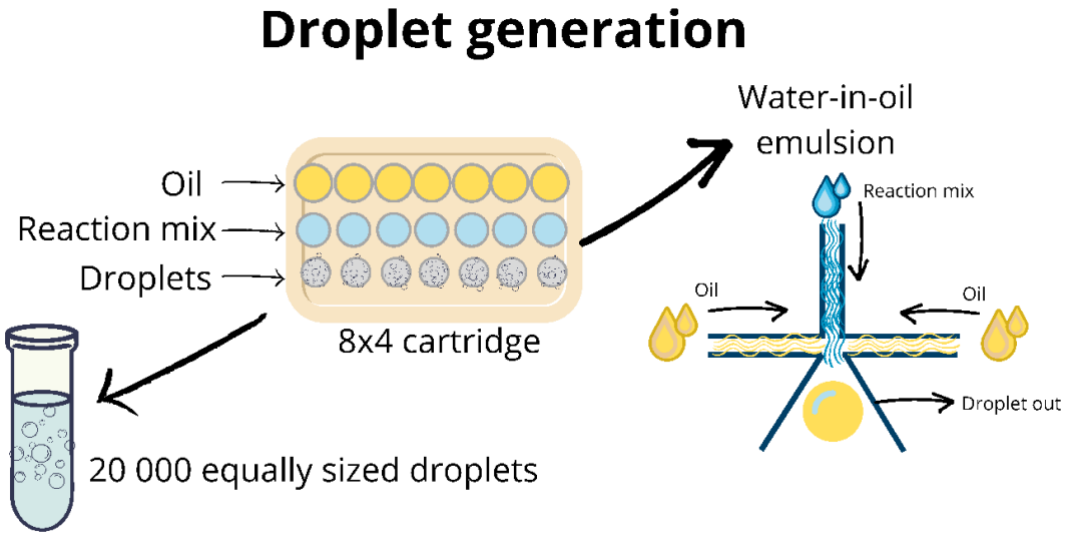

Figure 3. *Schematic figure showing the set-up of oil and aqueous reaction mix and the mechanism of water-in-oil emulsion for droplet generation.*

3.3.3 Preparation for PCR

The newly made droplets were then transferred into a single column of a 96-well PCR plate. Again, to not disrupt the droplets, a special pipetting technique was carefully followed. An 8-channel multi-pipette was used, with 200 µl pipette tips. 40 µl of the droplets were slowly drawn up using an angle of 30-45 degrees, with the tips placed at the junction between the wall and the bottom of the wells and not to be flat against a surface at any given time. The droplets were then dispensed slowly into the wells of the PCR plate, near, but not touching the bottom. To avoid evaporation of the newly transferred droplets, the plate was immediately sealed with foil. This was done using foil compatible with the Bio-Rad PX1 PCR plate sealer and the QX200 droplet reader. The plate sealer was set to a 180 °C with a time of 5 seconds, before opening the tray door and placing the 96-well PCR, with its foil cover, appropriately on the support block of the machine.

3.3.4 PCR

The sealed 96-well plate was then placed in a standard thermal cycler machine with conditions specified in table 2, according to the Bio-Rad ddPCR Supermix for probes (no dUTP) protocol. The first step was denaturation, conducted at 95°C, thereby disrupting the hydrogen bonds separating the two DNA strands. Following denaturation, the temperature was lowered to 50-65°C in which the primers, which are present excessively in the reaction solution, anneal to the 3´ ends of each template strand. The temperature was then raised to the optimal temperature for the heat-stable Taq polymerase, usually around 75-80°C , whereby the Taq polymerase will attach on the 3'-end of the primers and start incorporating the free nucleotides of the solution into a new DNA strand as it moves along the template. The process produces two newly made DNA strands identical to the template, which further will be used as templates in the subsequent PCR cycles. Each cycle ends up with twice as much template DNA as was initially present and thus grows in an exponential manner, before it typically plateaus after 30-40 cycles. Each droplet undergoes PCR with amplification of the target DNA, if present.

Table 2. The thermal cycling conditions for the PCR reaction.

Cycling step	Temperature, °C	Time	Number of cycles
Enzyme activation	95	10 minutes	1
Denaturation	94	30 seconds	40

Annealing/extension	60	1 minute	40
Enzyme deactivation	98	10 minutes	1
Hold	4	infinite	1

3.3.5 Droplet reading

The probes used in the reaction were bound to both a fluorophore and a quencher, therefore initially not giving any signal. However, Taq polymerase will cleave the probe whilst synthesizing new DNA from the target strand, thereby freeing the fluorophore (figure 4). Droplets with the target DNA exhibited fluorescence signals whilst the droplets without did not. The level of fluorescence was detected and measured in the Bio-Rad QX200 droplet reader, which was connected to a computer running the QuantaSoft software. Based on the level of fluorescence in each droplet and a threshold limit which is set in QuantaSoft, each droplet was deemed either positive or negative for the target DNA. The proportion of negative droplets relative to the total number of droplets was used by QuantaSoft to determine the number of DNA copies in each sample. [119].

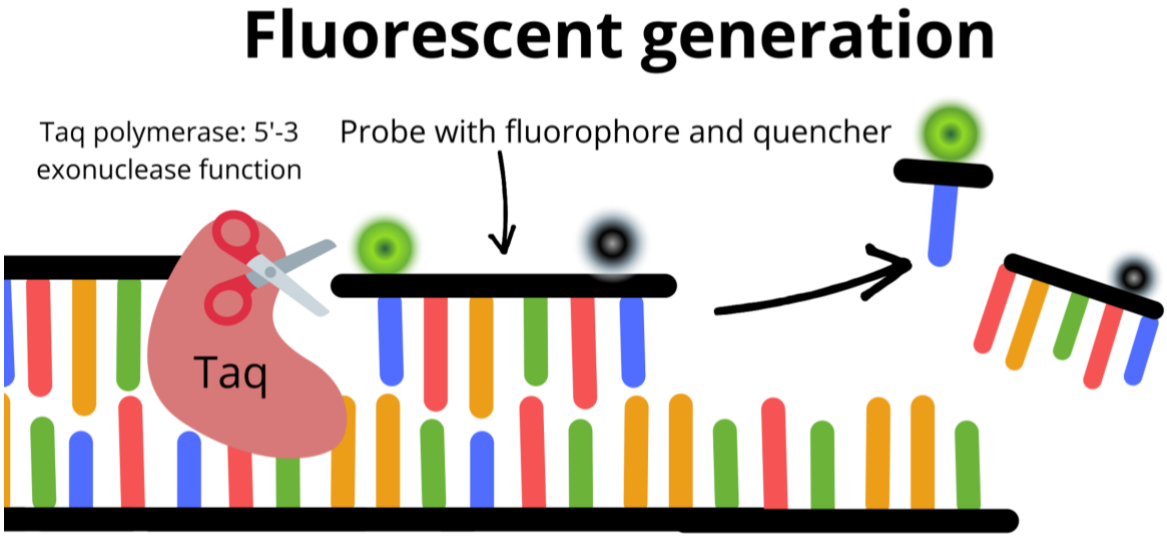

Figure 4*. Schematic figure showing how fluorescence is yielded when the probe binds to target DNA. The fluorophore will not emit any fluorescence whilst in proximity to the quencher, only when released, as happens when cleaved by Taq polymerase.*

3.4 Fluorescent in situ hybridization (FISH)

Before performing FISH, the paraffin had to be removed. Firstly, the slides were placed on a heat plate for 1 hour at 60°C for the paraffin wax to melt. For complete deparaffinization [120], the slides were treated with Histoclear, which highly dissolves the paraffin [121]. The Histoclear was then removed by a hydration ladder, consisting of 100%, 96% and 70% alcohol in which the slide was placed for two minutes each respectively. The slides were then left in tap water.

The paraffin- and Histoclear-free slides then underwent pretreatment, which consisted of a heating step and digestion step. Placing the slides in the Tris-EDTA buffer, the heating process was conducted in a pressure cooker at a temperature of about a 100°C C. The slides were then left to cool down on the bench, still in the buffer, for 20 minutes. The digestion step was then performed by adding protease directly to the tissue specimens, and subsequently placing it in a hybridization oven at 37°C for 1 hour. The slides were then taken out and the digestion was stopped through a dehydration ladder, immersing the slides for 2 minutes each, starting with 70%, thereafter 96% and finally 100% ethanol. The slides were thereafter left to air dry and were ready for probe application. 6 µl of the probe-mixture was applied to each of the samples and cover slipped immediately, the mixture consisting of hybridization buffer, purified H2O and the probes. The probes used were the Vysis DXZ1 and DYZ3 probes (Abbott Molecular Inc., USA), being specific for the X and Y chromosome respectively. Post probe-application, the slides were left on a heating plate set to 80°C for 10 minutes for denaturation. The coverslips were thereafter sealed to the slides with rubber cement, to avoid evaporation of the probes during hybridization, which was performed by placing the slides in a hybridization oven at 37 °C overnight. After hybridization, the coverslip and rubber cement were removed, and post hybridization washes were carried out. The latter consisted of wash buffer II for 5 minutes, wash buffer II at 74°C for 2 minutes and lastly wash buffer I for 1 minute. The slides were then dried and mounted with DAPI, before being cover-slipped and sealed with nail-polish. DAPI stains cell nuclei by binding to the AT-regions of DNA. Upon binding, its blue fluorescence increases about 20-fold, creating a strong signal allowing for nuclei detection [122].[122].

The slides were then ready for inspection under the microscope.

3.5 FISH analysis

For analyzing FISH results, the Leica TCS SP8 STED confocal microscope was used, a part of the core facilities of the Oslo University Hospital. Being necessarily connected to a computer, the LAS X software was used and set up with the appropriate wavelengths according to the fluorescent probes by the help from an employee at the core facility. An oil objective was used as these have the highest magnification ability for the type of specimens we tested. The confocal microscope is also paired with a conventional fluorescence microscope which, as it has filters appropriate for detecting DAPI fluorescence, is used for maneuvering around the tissue and finding an area of interest prior to confocal scanning. For each tissue sample, an area rich in nuclei was identified before setting up the confocal view through the LAS X software. A z-stack was then created before starting the confocal scan.

4 Results

4.1 Droplet digital PCR

ddPCR was performed on atherosclerotic plaque tissue from 15 women and one man. None of the female samples showed scores for the Y-chromosome (Figure 5). Chromosome 1 was detected in all samples at calculated ranges between 3000-6000 copies per 20µl sample. In the male sample, there were 1809 droplets containing at least one Y-chromosome and 3795 droplets with at least one copy of chromosome 1 (figure 6). This was calculated by the QuantaSoft software to a total of 2480 Y-chromosomes and 5520 copies of chromosome 1 in the sample. The non-template control showed no hits for either chromosome.

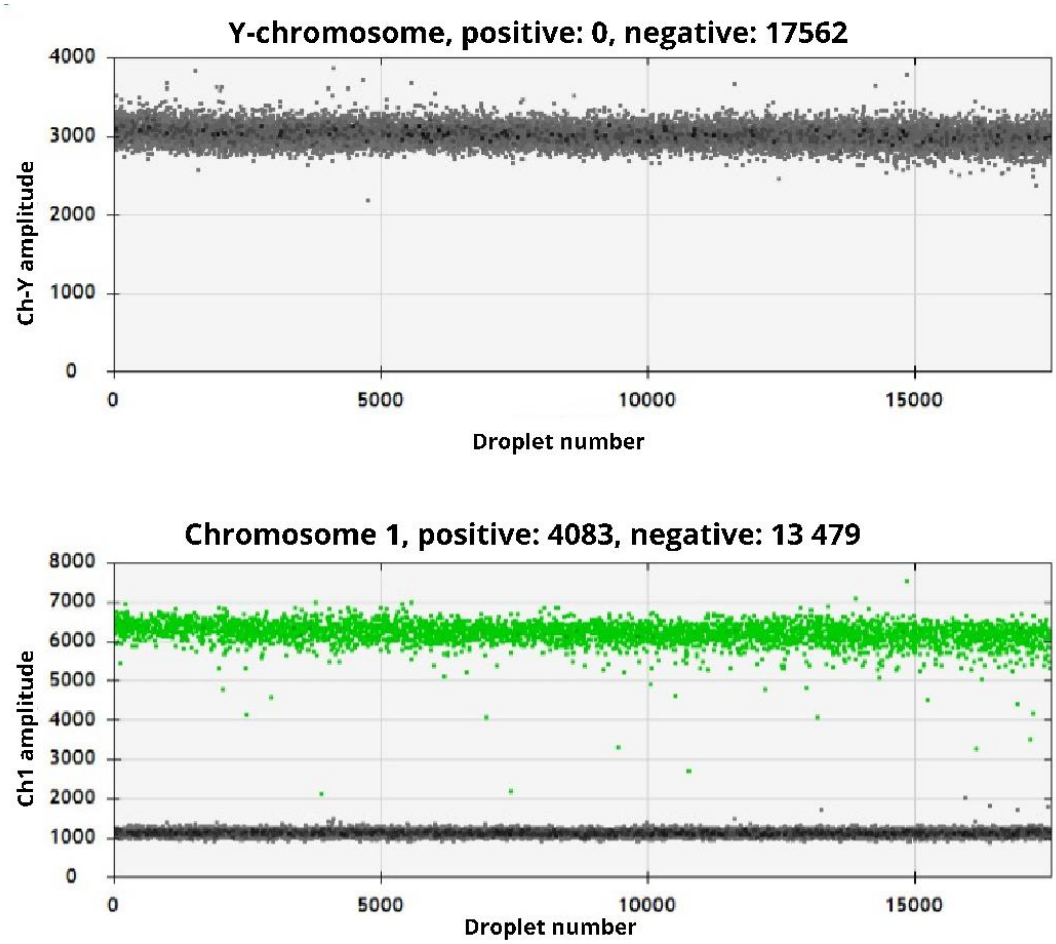

***Figure 5**. Plots from ddPCR on female atherosclerotic tissue. The amplitude measured for droplet is plotted against the event number, ie. droplet number. If a droplet has an amplitude higher than the set threshold, it is registered as positive (given a color), otherwise as negative (black). The number of positive and negative droplets for each target chromosome can be seen above the plot. The top plot is for the Y-chromosome and the lower plot*

for the chromosome 1. Number of Y-chromosome copies calculated per 20 µl: 0. Number of chromosome 1 copies per 20 µl: 6220.

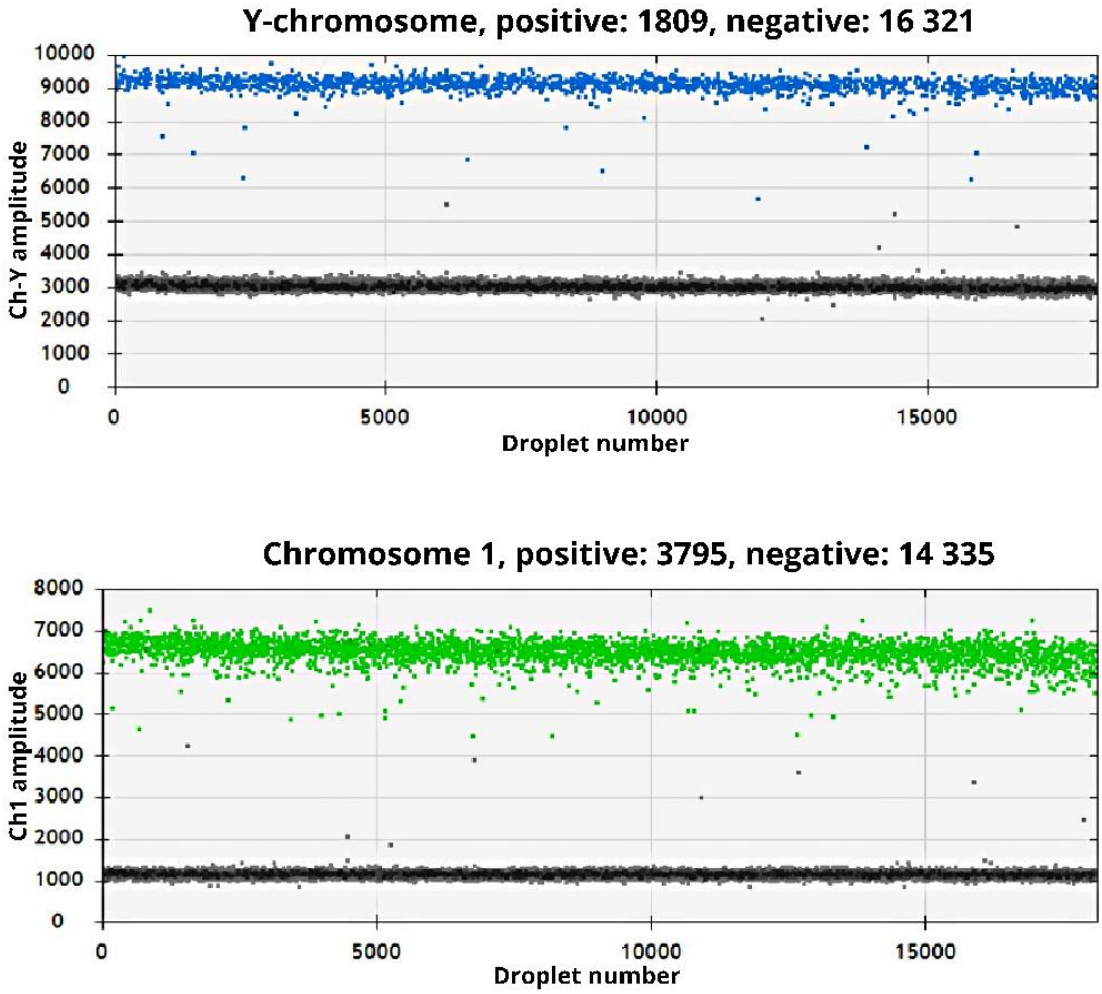

***Figure 6**. Plots from ddPCR on male atherosclerotic tissue done. Number of Y-chromosome copies calculated per 20 µl: 2480. Number of chromosome 1 copies per 20 µl: 5520.*

4.2 FISH

FISH and further analysis by confocal microscopy were performed on one decidual and two placental tissues from another three different women, followed by analysis of the distal and central parts of atherosclerotic tissue from five different women. The magnification of the confocal microscope was set to 40x.

4.2.1 FISH in placental tissue

Looking at the histology of the placenta, described by others [123] [124], it seems highly likely that the structures seen with FISH were cross-sections of placental villi. Placental cross-section is described with the surrounding multi-nucleated syncytium (syncytiotrophoblasts) and a few underlying cytotrophoblasts, with fetal blood vessel cells and stromal cells in the center. In the placenta of the female fetus, only X probe-signals (green) were identified (Figure 7). The syncytium can clearly be detected by the multitude of X-chromosomes in the fused outer lining of the structures (figure 7). Cytotrophoblasts are difficult to detect given their low presence in term-placentas [123]. The clear single cells with two nuclei seen are likely cells of fetal blood vessels. In the male placenta, signals from the X- (green) and Y-chromosomes (red) were visible in all cells and in the syncytium layer (Figure 8).

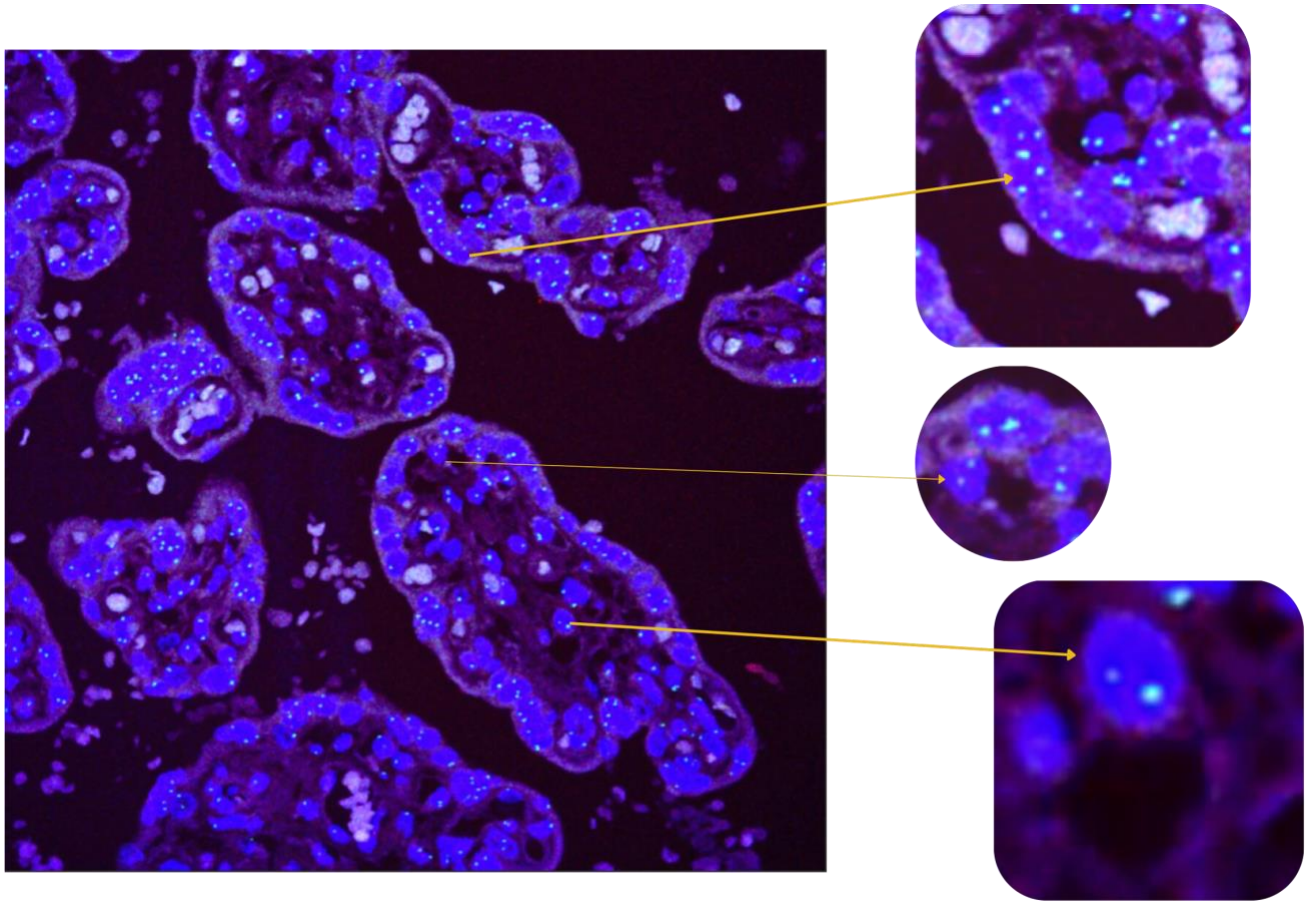

Figure 7. *Female placental tissue after FISH assay. The green, fluorescent dots show hybridization of the X-chromosome probe to the target. The larger blue areas show the cell nuclei. From the top, first arrow shows multinucleated syncytium. The two bottom arrows likely show fetal endothelial cells due to their mononuclear character (two signals within the nucleus) and their location adjacent to a blood vessel, seen as a circular cavity.*

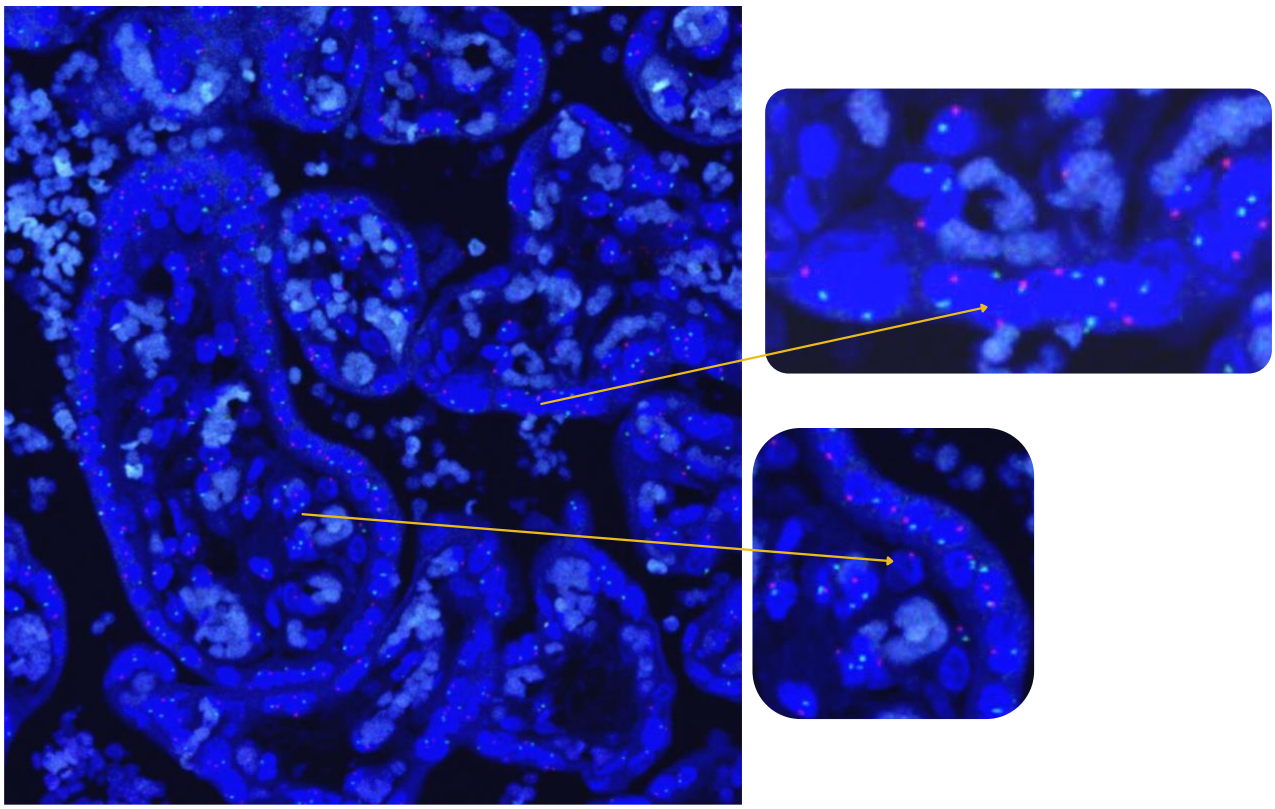

Figure 8. *Male placental tissue after FISH assay. The small green, fluorescent dots show hybridization of the X-chromosome probe to the target, likewise with the red dots and the Y-chromosome. The blue larger areas show the cell nuclei. From the top, the first arrow points towards syncytiotrophoblasts. The second arrow points towards what may be a cytotrophoblast given its location directly under the syncytium and its mononuclear character.*

4.2.2 FISH of decidua basalis

In decidua basalis from a pregnancy with a male fetus, extra-villous trophoblasts were identified with positive signals for both the X- and the Y-chromosomes (Figure 9). For these experiments, the X-chromosome probe was red, and the Y-chromosome probe was green. Immunohistochemistry staining was previously applied to adjacent tissue slides to identify areas with extra-villous trophoblasts – positive for CK7.

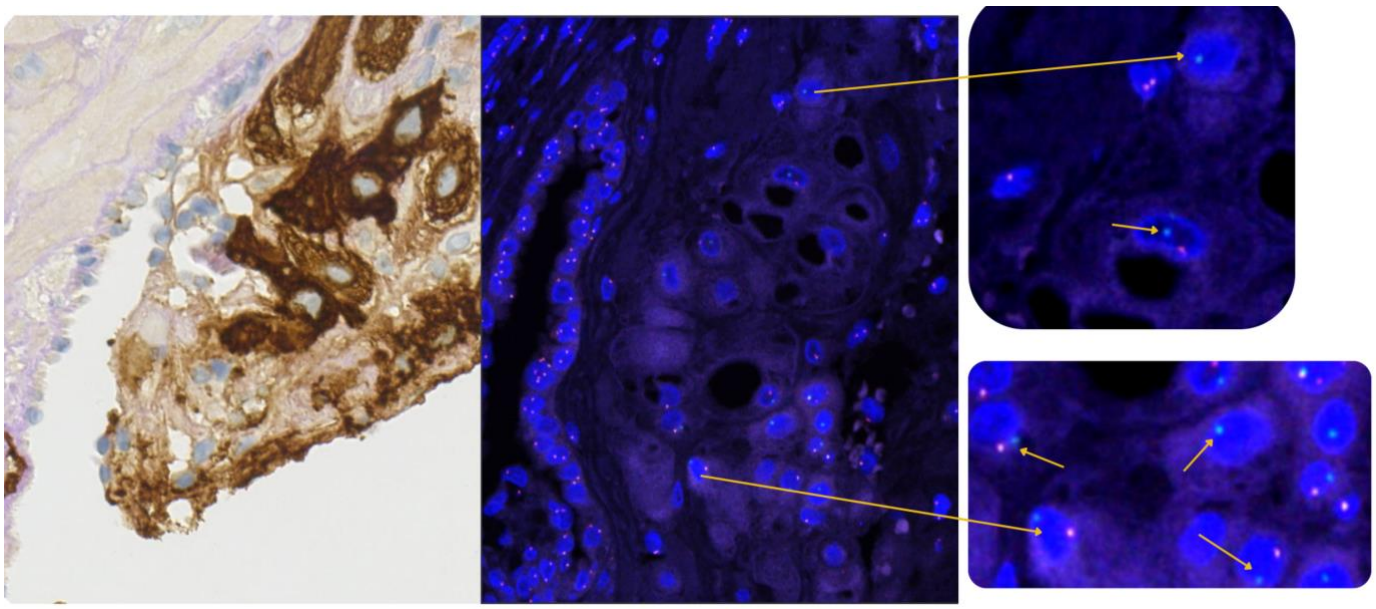

Figure 9. *Same area of decidual tissue from a pregnancy with a male fetus, stained with CK7 to the left and FISH analysis to the right. In CK7 staining, trophoblast cells can be identified from the brown color and a light blue center. Cells in the same region were identified as male cells using XY-FISH through detection of green signals (Y-chromosome in this case), shown with arrows. Cells with double-red signals represent maternal cells.*

4.2.1 FISH of atherosclerosis

Of the 5 central and 5 distal atherosclerotic samples analyzed, 9 were positive for the X-chromosome signal (green) whilst one gave off no detectable signal (figure 10, left). The quality was seen to vary between samples from the same patient and within regions of the same sample, both regarding hybridization-signal strength and the amount of background fluorescence. Of the 4 female samples, one gave signals for the Y-chromosome (red) together with the X-chromosome, within a DAPI-stained nucleus (Figure 11). In the male plaque, several nuclei stained positive for both the X- and the Y-chromosome (Figure 12).

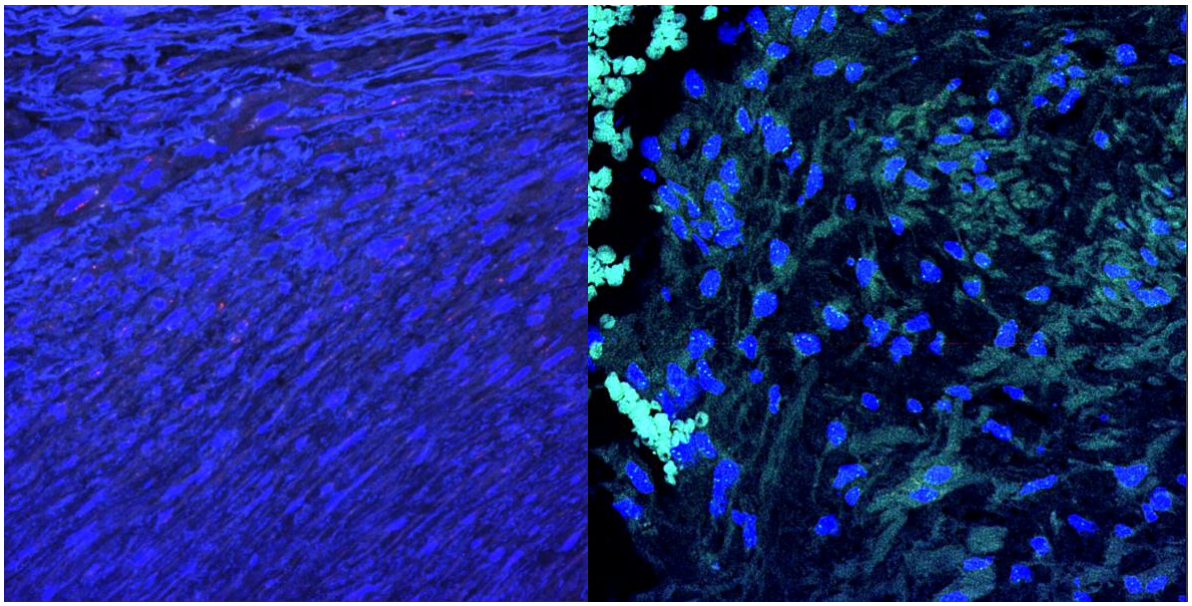

Figure 10. *FISH analysis of atherosclerotic tissue from the same patient. Central part, undetectable green signals and invalid red signals, to the left, and central part, with detectable valid green signals, to the right.*

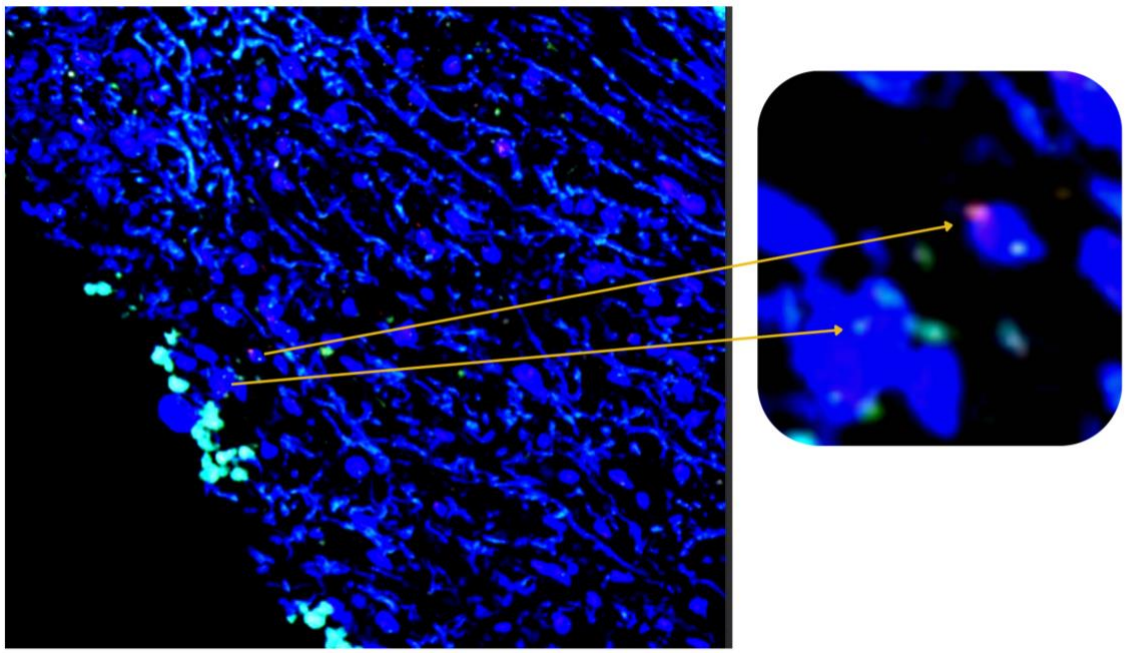

Figure 11. *FISH on atherosclerotic tissue. One clear red signal detectable indicating the presence of a Y-chromosome, top arrow. Bottom arrow showing a female cell with two green signals, indicating two X-chromosomes.*

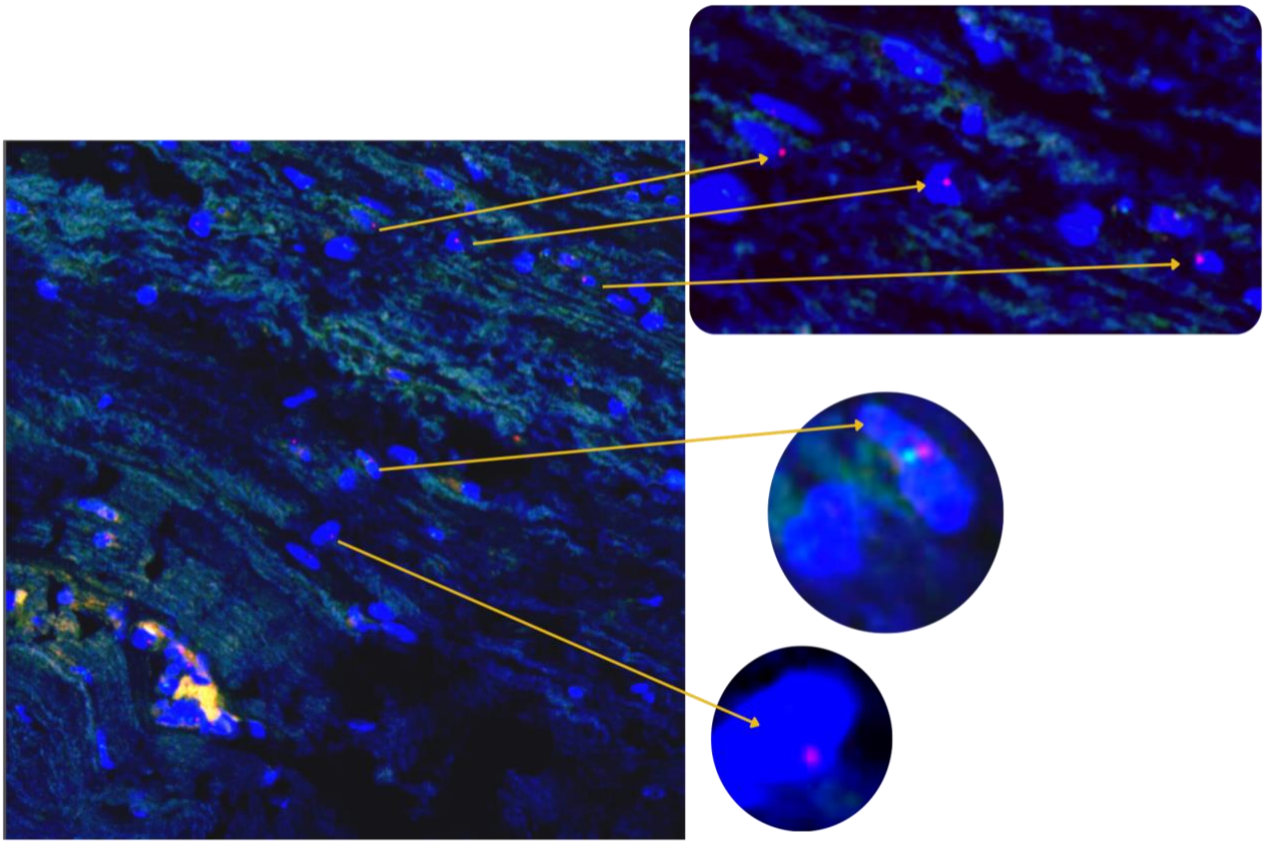

Figure 12. *FISH analysis of atherosclerotic tissue. Several valid red signals detectable, shown with arrows.*

5 Discussion of methods

5.1 Tissue handling

The frozen tissue used for ddPCR was cut and grinded into powdered tissue, a process which is kept cool by liquid nitrogen. Freezing with liquid nitrogen allows for extremely rapid freezing which is advantageous for several reasons. These include all chemical reactions coming to a halt, no significant diffusion of cellular components and, lastly, that fixation of tissue is achieved at much faster rates than with chemical fixation [125]. The crushing of tissue requires temperatures at maximum -19 °C and is therefore a process characterized by efficiency and focus to prevent melting. Another effective alternative to liquid nitrogen is immersing the tissue in dry ice [126] [127], which was used for storing the frozen tissue, in tubes, prior to grinding, and cooling of the metal mortar.

5.2 DNA isolation

The breaking open of the cells, cell lysis, is a necessary step before isolating the DNA from the sample. Cell lysis can be performed through chemical, mechanical or enzymatic action. A mechanical way would possibly involve grinding, applying high pressure etc. however these methods may also damage the DNA and thus may not be suitable for DNA extraction. However, in the presence of liquid nitrogen the DNA remains protected through inactivation of harmful chemicals and enzymes [128]. Chemically, detergents can be applied thereby solubilizing the lipid membrane of the cells t causing gentle cell lysis. Detergents also exhibit an inhibitory effect on DNAses and a denaturing effect on proteins. An example of an enzymatic lysis is the use of the lysozyme enzyme to break down the cell wall, thus relevant for plant cells or bacteria [118]. The exact composition of ATL used in this experiment is not known as it is not provided by the manufacturer, but likely has the effect of a detergent.

Accompanying cell lysis is the removal of proteins which is achieved by the addition of proteinase K, the benefit being that it is a broad-spectrum proteinase, which will digest any proteins present [124]. Following the Qiagen protocol, the cell-lysis mixture should be incubated at 56 °C.

Cell lysis was first performed on 8 atherosclerotic frozen tissue samples. They were left to incubate overnight with 20ul proteinase K and 180ul buffer ATL. However, by the following morning the complete lysis was deemed incomplete as small bits of tissue could still be detected in the fluid through visual examination. Particularly the presumed calcified parts, seen as white "bony" bits, showed resistance. This led to doubling the amount of ATL and proteinase K. Following this modification, the tubes were left to incubate for 1h at 56 °C after which the tissue in the tubes was deemed sufficiently lysed, despite a few solid bits still being present. The reduced time for incubation allowed for lysis of all 16 tubes in 2 hours.

5.3 ddPCR

PCR was invented in 1984 and considered a revolution in the science world [129]. It is a laboratory technique capable of rapidly amplifying specific DNA sequences as much as a billion-fold. As the result of the reaction will leave you with millions or billions of copies of the target DNA compared to just the initial few, it makes for an extremely easier further investigation of the specific sequence. Since its debut, it has been used for prenatal screening, genetic cloning, and diagnosis of infectious diseases. The main components necessary for the biotechnological method include a template, primers, free nucleotides and finally, the DNA polymerase, which is the main driver of the replication reaction [130]. The ddPCR-supermix provided by Bio-rad contains all the components necessary for the reaction apart from template, primers and probes and is specifically formulated to work with ddPCR according to the manufacturer's application guide.

Both conventional PCR and quantitative PCR (qPCR) have been used for detecting fetal microchimerism [131, 132], with qPCR holding the benefit of being able to quantify the initial amount of target-DNA in the sample rather than just confirming its presence [133]. Further, digital PCR has been shown to have a detection-limit 10 times lower than that of qPCR, in addition to giving more precise and less variable results [134] [135] [136]. Examples of cases where digital PCR has been proven to detect rare target sequences include detecting fetal DNA in maternal blood plasma [137] and viral genes in bacterial cells [138]. Thus, digital droplet PCR was deemed appropriate for the detection of fetal DNA within the maternal atherosclerotic tissue samples. Digital PCR is based on partitioning the sample into small micro-units and in digital droplet PCR these small partitions are the droplets made. With each droplet either scoring positive or negative for the target molecules, this also makes it less error prone as

detection is only needed between two levels: positive or negative based on a threshold level of fluorescence [139].

The number of droplets formed in each sample can always be calculated by adding the number of positive droplets and negative droplets, for one target, together. None of the samples yield a droplet sum of 20 000 and thus it is obviously a theoretical optimum. Considering partition statistics, each droplet can be seen as an independent entity and the probability that one target molecule, for example one Y-chromosome copy, is present in each of those droplets is 1/n, where n is the number of droplets. If there were 5 droplets, the probability of one target molecule having "fallen" into each of them, i.e., success, would be 1/5. This trial is repeated for each target molecule and follows a binomial distribution, which gives the probability of number successes per partition, i.e., probability that a selected partition has x copies of the target molecule. When n is large, the binomial distribution can be approximated by the Poisson distribution which is used to calculate the sample concentration through the number of empty partitions. Therefore, the exact number of 20 000 partitions, droplets, is not crucial but it has to be a high number. The number of targets also must be lower than (n*ln n) for the quantification to be possible. [139].

DNA polymerase I is the enzyme which carries out DNA replication in human cells. However, PCR is a process which involves temperature fluctuations and thus requires a thermostable DNA polymerase. The polymerase used was the Taq polymerase, originally discovered in hot spring living bacteria [130]. PCR is also a reaction which exploits the nature of DNA including its melting point, the complementary base-pairing and the ability to spontaneously reanneal. The process involves three processes which are all temperature dependent and therefore can be carried out in a thermal cycling machine.

From a test-run of ddPCR previously conducted on atherosclerotic female tissue, performed in January, QuantaSoft calculated that the sample held 128 copies of Y-chromosome and 31780 copies of chromosome 1, as seen in figure 13.

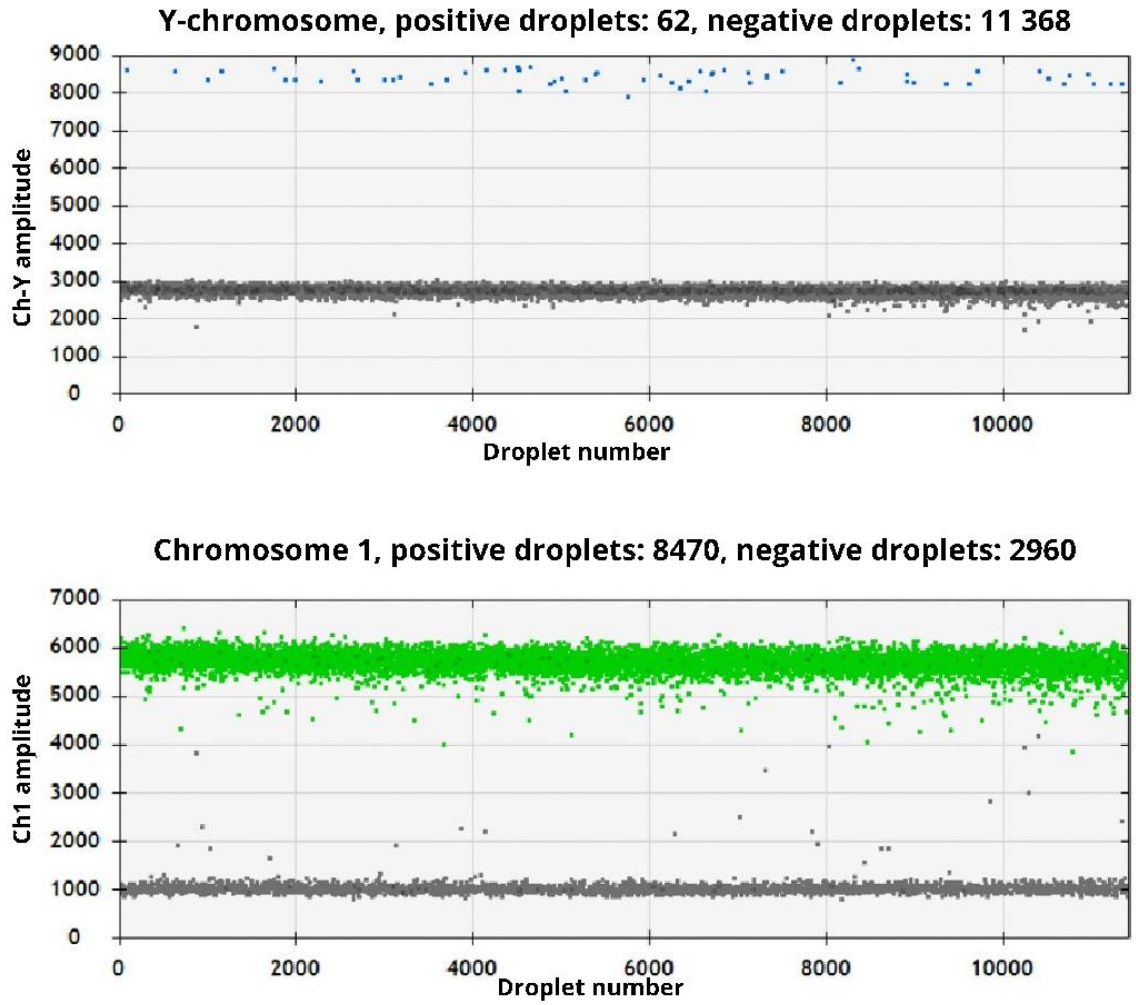

Figure 13*. Plots from a ddPCR test-run of female atherosclerotic tissue done in January. Each droplet is plotted with its amplitude against droplet number. Top plot representing droplets checked for the Y-chromosome, the bottom for chromosome 1. Positive and negative numbers referring to positive and negative droplets respectively.*

Because every cell has two copies of chromosome 1 it is appropriate to divide this number by two to get an approximate cell number, thus giving 15 890 cells. As male cells only have one Y-chromosome, 128 Y-chromosome copies translates to 128 male cells in the sample. The proportion of male cells relative to female cells was then calculated to be 0.008 or 0.8%. It was suggested at the time that cross-contamination could not be excluded as the source of the male DNA, as the mortar, used in the process of grinding the tissue into powder, was not extensively washed between samples, only cleaned while cooled with isopropanol. Therefore, for the crushing of the atherosclerotic tissue in our experiment, an extensive washing process was conducted to remove all trails of male DNA before the first sample, and between samples.

The defrosting of the metal mortars, necessary for the washing process, needed to happen slowly to avoid shape disruption as each consisted of a bottom and a lid which fit together, in

addition to a hole through the lid in which the crushing pole was placed. Because there were three metal grinders, only three tissue samples could be powderized rapidly in one go before the decontamination wash could be carried out. This therefore became a major rate-limiting step in the DNA isolation process and several days were needed for the grinding and crushing of the 16 tissue samples. Nilsson et al. have tested several different treatments for DNA removal through depositing blood on different surfaces, followed by DNA removal treatment and the resulting DNA yield from the surface [140]. On the metal surface, they showed that Virkon resulted in the least amount of DNA yield with only 0.1% of the non-treatment of control. According to manufacturer's instructions, Virkon needs to sit for 10 minutes before being washed off with water. Considering this, it seems that the rate-limiting step of slow defrosting before washing was indeed necessary. An alternative DNA removal method that could be carried out whilst the grinders remained frozen is exposure to UV light for 20 minutes, however this as a stand-alone treatment gave a 12.1% DNA yield of the non-treatment control and thus is not sufficient [135].

5.4 FISH

Fluorescence in situ hybridization was first developed in the early 1980's and advanced the field of cytogenetics: the study of the structure, properties, and potentially changes, of chromosomes [141]. The basic concept is the use of fluorescent DNA probes that bind to specific target sites on the chromosomes in the cell nucleus, which gives colored signals that can be detected through a fluorescent microscope. Abnormal changes in DNA include translocation, deletion and duplication and the detection of such abnormalities are often used in diagnosis, prognosis, and prediction of certain diseases, for example cancer. One example is the diagnosis of lung cancer in which multiple copies of chromosome 6 is common, and therefore the malignant cells can be detected using FISH with a probe specific for that chromosome. With healthy lung cells harboring two copies of chromosome 6, one from each parent, the malignant cells may have 3 or more copies which will show up as the same number of fluorescent signals in the microscope [142]. Similarly, it can also be used in prenatal screening for detection of aneuploidies, for example trisomy 21 (Downs syndrome) or multiple copies of the sex chromosomes. Although the examples mentioned regard whole chromosomes, the target of interest may also just be a single locus [143]. The application of FISH to genetic

abnormalities has broadened along with the numerous discoveries of disease related genes [144].

5.4.1 Protein digestion and fixation time

Some of the atherosclerotic tissue samples never gave off any fluorescent signal or gave weak signals with a lot of background, such as seen in figure 10(left) and 11. Low or weak probe signals may imply insufficient digestion, in addition to intact proteins generating autofluorescence. However, if digestion is left too long this can destroy tissue morphology and lead to increased background fluorescence. Therefore, in each FISH experiment this step is often a process of trial and error, finding the optimal time for the sample of interest [145, 146]. The protein digestion in this FISH experiment was carried out by ISH protease 3. Proteases cleave the covalent bonds between proteins and their specificity varies, with some only cleaving a particular bond on a particular protein, others cleave in a highly promiscuous manner [147]. ISH protease 3 in this FISH experiment was a type of serine endopeptidase, is non-specific and cleaves all bonds [148]. The time it takes for sufficient digestion varies and may depend on how long the tissue has been fixated for, tissue type, protease concentration and tissue-section thickness [149]. The same protocol was done on all the placental, decidual and atherosclerotic samples, clearly showing the difference between tissue types as decidual and placental tissues, figures 7-9, give stronger hybridization signals with less background than the atherosclerotic tissues, figures 10-12.

In a previous publication where FISH was used to search for microchimerism in several different tissues, 5–10-minute protein digestion was sufficient for skin samples, whereas 10-15 minutes was needed for heart tissue [150]. The digestion time by the protease is the step that differed in the final protocol compared to many others, letting the protein digestion work for 1h compared to 10-15 minutes [150, 151] [104]. However, digestion time was suggested by a competent employee at Oslo University Hospital and disruption to tissue morphology was not noted. Furthermore, as the main purpose of protease treatment is to break the covalent bindings made by the formalin the tissue was once fixed in, it might be that the necessary digestion time is influenced by the fixation period. Few of the articles describing fluorescence in situ hybridization on FFPE-tissue sections mention the length of time the tissues were fixed for and rather focus on tissue-type. The impact of fixation time is demonstrated in an article optimizing an in-situ hybridization method [152]. They had FFPE-samples that were of different ages and

ran several optimalization steps to achieve successful assays. They concluded that all samples older than 1 year needed protocol optimization and that some samples were simply deemed unsuccessful, the latter possibly being due to a long archival period and suboptimal fixation issues such as temperature.

In order to stop digestion, a dehydration ladder was used as ethanol is shown to inhibit protease activity [153]. Dehydration is also necessary prior to probe-application which directly follows to prevent probe dilution and to ensure complete absorption of probe mixture to tissue [154]. Denaturation was then conducted at 80°C as DNA has a melting point of 72.6 °C [155]. This will lead to breaking of the hydrogen bonds holding the double stranded DNA together, thereby making the target sequences readily available for the probes. Hybridization happens upon reannealing of DNA, which occurs in the hybridization oven at 37 °C. The hybridization buffer the probes are applied with largely consists of formamide which has a destabilizing effect on DNA [156]. Because there is a chance the probe has bound to not only the target sequence, but also other regions of the genome, during the hybridization step, post-hybridization washes are necessary to elude such non-specific binding and unbound probes. The washes consisted of Sodium-Saline Citrate (SSC), NP-40 and water. SSC contains positively charged sodium ions which interact electrostatically with the negatively charged DNA backbone. Upon this interaction, the otherwise repulsive forces of the negative DNA backbone is masked by the positive ions, thereby increasing stability of the DNA [157]. The high-temperature wash will ensure denaturation of poorly matched probes to DNA whilst the probes of high complementarity remain as these are more stable [158].

5.4.2 Detecting fetal microchimerism

The basis of hybridization between probe and target DNA lies in the chemical nature of DNA. It consists of two long polypeptide chains bound together by intermolecular hydrogen bonds. One nucleotide consists of a sugar ring, deoxyribose, with a phosphate group and base attached and the nucleotides are further covalently linked between the sugar of one nucleotide and the phosphate group of the next. The bases of DNA are Thymine, Adenine, Cytosine, and Guanine commonly abbreviated with their first letter T, A, C and G respectively. It is the order and combination of these bases that give specific DNA sequences and may encode specific proteins. Further, it is the bases that are involved in the hydrogen bonding between two strands of DNA, however hydrogen bonds cannot arise between any combination of bases within the DNA

because of the chemical and spatial properties of each base. Adenine will always pair with thymine, and cytosine will always pair with guanine. Consequently, this creates the complementarity seen in DNA, if one 4-nucleotide portion of DNA reads “ATTC” its complementary counterpart will read “TAAG”. [159]. This is the mechanism that is exploited in many methods of DNA detection utilizing DNA probes, including FISH and PCR. The probes utilized must thus be complementary to the target DNA and choosing an appropriate one is consequently one of the most important steps when performing FISH. A wide range of probes are available varying in size from 1 to 10 thousand bases, being specific for repeated sequences across the genome, a gene locus or even a whole chromosome. For the detection of fetal microchimerism in both ddPCR and FISH, male cells were targeted, being of presumed fetal origin from a previous pregnancy with a male fetus. [160]

The difference on a chromosome level will be that the male cells harbor the XY karyotype, contrasting to the XX maternal cells. In FISH, both the probes used are therefore specific to regions found on the sex chromosomes. The probe targeted to the X chromosome is an alpha satellite probe, the alpha satellite DNA being a repeated sequence of the centromere of the chromosome. The centromeric regions of chromosomes are highly conserved and play an important part in cell division. Further, the probe is specific for these repeated regions on the X chromosome in the Xp11.1-Xq11.1 region, as the alpha satellite organization differs between chromosomes [160]. Xp11.1-Xq11.1 denotes chromosome positions, X being the chromosome, p or q meaning the long and short arm of the chromosome respectively, and 11.1 referring to band and sub-band. Xp11.1 thus means chromosome X, short arm, band 11 and sub-band 1 [161]. As the probe will hybridize to multiple copies of the alpha satellite sequence, this yields a strong signal [162]. The probe is attached to a fluorescent marker emitting green light. The Y-specific probe is also an alpha satellite probe spanning the Yp11.1-q11.1 region and is attached to a fluorescent marker emitting red/orange light [59].

5.4.3 The Y-chromosome target: Benefits and limitations

Despite being a useful target, the Y chromosome does not come without limitations. The standard XY-FISH bears the risk of false positives in which female cells give off a Y-chromosome signal. This was shown in a study in 2005 attempting to use FISH to determine the fetal sex in pregnant women, however they were able to up the specificity from 69.4% to 89.5% by substituting the X-probe with a probe targeting a different region of the Y-

chromosome. In this case it was only the cells giving off a double Y-signal which would be deemed male and the ones giving off a single signal would be deemed female false-positive. In 31.6% of the pregnancies with a female fetus, male cells, according to XY-FISH, were detected and therefore deemed false-positives, but they do not exclude the fetal cells from previous pregnancies being the cause [163]. However, another study demonstrated the ability to detect one male cell amongst 100000 female cells, thus being appropriate for the rare event that is fetal microchimerism. Further, in the same study no false-positive signals in pure female cell populations were detected [164].

Another obvious disadvantage with targeting the Y-chromosome is the lack of possibility of detecting fetal cells originating from a female fetus. As both the mother and the female fetus possess the XX-karyotype, the elegant way of distinguishing them from one another on a chromosome-level is discarded. Therefore, for this to be possible, probes which target the highly polymorphic Human leukocyte antigen (HLA) may be used [165]. This method is based on each individual harboring one HLA-allele from each parent and thus, the fetus will carry one allele which will match one of the mother's two alleles and one which will be foreign, inherited from the father: the non-shared allele. In cases where the allele inherited by the father is identical to the non-inherited allele from the mother, different polymorphic sites may be used. By targeting the non-shared allele, it would therefore be possible to distinguish between fetal and maternal cells. However, this requires genotyping of both the mother and child, making it a highly costly and demanding prospect [85]. Considering this, another great advantage of the X and Y-chromosome probes is that they are commercially available. This eliminates the heavy workload involving the design, preparation and labeling of specific probes as in the case of HLA-probes. Further, the protocol needs then not only be developed for the samples but also the probes; both the temperature and hybridization buffer components affect probe sensitivity and specificity [166]. The labor involved in This genotyping, design, labelling also applies for potentially using specific HLA-probes in ddPCR, as well as designing specific primers.

5.4.4 Confocal microscopy

Confocal microscopy is a technique based on conventional wide-field fluorescent microscopy but with a few modifications. With the basic fluorescence microscope, fluorescent and white light from the microscope light source is sent in, hits the dichromatic mirror and is directed towards the specimen through an objective, which focuses the light. In the specimen, there may

be fluorophores. Fluorophores are molecules capable of absorbing light, thereby being excited to a higher energy level, before coming down to a lower energy level just a few nanoseconds later. In this process, light is emitted at a longer wavelength, i.e., holds lower energy. The dichromatic mirror works as a normal mirror with shorter wavelengths but allows longer wavelengths to pass through. Thus, the light from the light source gets reflected onto the specimen, but the emitted light, with a longer wavelength, passes through. So, the emitted light from the fluorophores, the fluorescence, reaches the detector which can be either an eye or a camera. Often, excitation and emission filters are also added to enhance the selection of appropriate wavelengths. For example, if the molecule of interest is bound to a fluorophore that absorbs light in the blue-wavelength range and emits in the green wavelength-range we want excitation and emission filters that are specific for these ranges. The challenge is that even though the area of focus by a conventional microscope is small, light/fluorescence is also being emitted from other out of focus areas, which often gives a blurry view or photo. This is particularly true for thicker specimens. [167] [168] [169]

The confocal microscope thus has two major modifications to facilitate a clearer image, the first being the source of excitation; instead of light, there is a laser. The most important consequence with this change being that lasers work as a point source of light, and therefore differ from the conventional fluorescence microscopes in that they do not illuminate the whole specimen at once. The second change is done to the emitted light from the specimen; instead of collecting all the light, an adjustable aperture diaphragm is used, known as the pinhole. This functions to physically reject the emitted light from areas outside the focus area of the objective. This is done through the light from the laser hitting the dichromatic mirror, the laser is monochromatic, so no filters are needed, being directed to the objective and hitting a small area of the specimen. The specimen will then emit light which will pass through the dichromatic mirror, hit an objective with the same focus point as the one which focuses the laser, and the pinhole will then only allow the focused light from the objective to hit the detector. The signals the objective collects which are out of focus will be physically rejected by the edges of the pinhole. Because the confocal microscope uses point focus, for an image to be created the specimen must be scanned pixel by pixel in an x and y axis matter, whereby the fluorescence is recorded in each coordinate. Further information can be conducted from a confocal scan from setting different z-coordinates, that is, 2D scans (x and y axis) from different focus positions and stacking these, scanning from different focus positions together [167] [168] [169].

5.4.5 Alternatives for analyzing FISH

Manual detection of FISH signals using a conventional fluorescence microscope using different appropriate filters often requires expertise, is biased and time consuming. In one study they report that one slide analysis, focusing on 200 cells typically takes 30-60 minutes [170]. However, the study was aimed at detecting chromosomal abnormalities within the nuclei, and the analysis of each nucleus was likely to be more labor-intensive than that of XY-FISH, where the analysis of each nucleus is binary. Fetal cells were detected in both the appendix and C-section scars using conventional fluorescence microscopy with different filters, whereby no mention of difficulties is made by the authors [104, 171]. On the contrary, confocal microscopy is focused on a very small area and the scanning time for each image is relatively slow. This becomes particularly relevant and disadvantageous when the area of the potential target is unknown and is expected to be an extremely rare event. Solutions to overcome manual detection techniques include an automated scanning system such as whole-slide imaging. However not commonly used for FISH slides yet, successful methods have been developed which also include the stacking in the z-direction and thus carry the same benefit as confocal microscopy [172, 173]. The signals from the digitally scanned images can then be counted manually or automatically, with the latter being conducted in a significantly shorter amount of time. However, the automatic method has been shown to be highly erroneous and its algorithm and application therefore needs to be developed according to the type of specimen being examined [172, 173]. An additional benefit of digitally scanned images is the opportunity to easily view them in parallel with H&E scanned images, showing tissue morphology [174], or other markers, for FISH comparison in a computer software program.

6 Discussion

In the present study, a protocol for both FISH and ddPCR for investigating the presence of Y chromosome-positive cells within female tissues, was developed. Using ddPCR, no Y-chromosomes were found in the 15 female atherosclerotic samples studied (though Y-chromosome was present in a test-sample run but suspected of male DNA contamination). The findings using FISH on placental tissue were in line with the known multinucleated nature of the syncytiotrophoblast. In the decidua basalis sample from a female pregnancy, successful identification of several XY-positive cells in CK7-rich areas, confirming that these cells were fetal extra villous trophoblasts and not maternal epithelial cells. FISH staining of atherosclerotic tissue gave results of varying quality. Interestingly, a possible XY-positive cell was found in one of the female atherosclerotic plaque samples, possibly indicating the presence of a male cell of fetal origin.

Even though no Y-chromosome copies were detected in female atherosclerosis using ddPCR following implementation of the decontamination protocol, it cannot be concluded that there were no male cells present in the tissue, the reasoning being the number of cells in the ddPCR run. Chromosome 1 was present at values around 6000 copies per sample, ie. 3000 cells. If one male cell of these cells was found, it would be a ratio of 0.0003. The frequencies at which fetal microchimerism are present in both blood and tissue range from 1 in 1 000 000 to 4900 per 100 000 [175]. Although fetal microchimerism has been found as high as 0.049 previously if the real ratio of fetal to maternal cells in the atherosclerotic tissue is as low as 1 per million this would be missed when analyzing only 3000 cells. The problem could be solved by harvesting more DNA from the tissue, thereby yielding a higher initial concentration in the samples which will allow for higher DNA concentrations in the reaction mixes. The input of 20ng into the reaction mix is not a set amount provided by the protocol, but the maximum input possible with the initial concentrations being generally low. The protocol states that the input must be <66ng (or up to 330ng with restriction enzymes), however if the input was set to 60ng, approximately 22ml of the sample with 2.73 ng/µl initial concentration would be needed. As the reaction sample needs to have 12 ml of supermix, primers and probes, an additional 22ml would result in 34ml reaction mix, 10ml above the final volume limit provided by the protocol.

Increased DNA yield could be attempted by using a different DNA extraction method than the one in the Qiagen kit, which utilizes a silica membrane. An example of a different method is

using a method based on magnetic glass particles to extract the DNA. However, on an evaluation of extraction methods on atherosclerotic tissue the Qiagen DNeasy Tissue kit showed highest recovery rate [176]. Additionally, in another study comparing RNA extraction using a spin column technique and a magnetic bead-based technique, it was again concluded that the former resulted in increased RNA yield. The study also mentions the magnetic bead-based technique as lengthier, and difficult to perform compared to spin column technique [177].However, on an evaluation of extraction methods on atherosclerotic tissue the Qiagen DNeasy Tissue kit showed the highest recovery rate [174]. Additionally, in another study comparing RNA extraction using a spin column technique and a magnetic bead-based technique, it was again concluded that the former resulted in increased RNA yield. The study also mentions the magnetic bead-based technique as lengthier, and difficult to perform compared to spin column technique [175].

CK7 immunostaining is commonly used to identify extra-villous trophoblasts in decidua basalis [178] [179]. However, CK7 is also a marker of epithelial cells, lining uterine glands. Thus, CK7-staining alone cannot be used to identify trophoblasts invading uterine glands, termed endoglandular trophoblasts. In cases of a male pregnancy, co-staining decidua basalis with CK7 and the XY-FISH protocol used would indeed distinguish between endoglandular trophoblasts and maternal glandular epithelial cells. However, co-staining with another trophoblast marker, like HLA-G, is likely more practical [180].

Performing FISH on atherosclerosis proved more challenging than performing FISH on placenta and decidua basalis. This could be due to high variation in structural properties between plaques, and even between different regions of the same plaque. Therefore, although a possible XY-positive cell was identified within female atherosclerosis, this finding must be validated. Because XY-FISH has been shown to give false positives in previous studies [163, 181], a secondary FISH assay could be performed using two Y-probes [182], as previously discussed, or re-hybridized using the same probes with reversed colors. Only the cells being positive for two assays would be deemed true fetal cells. Further, because only a few cells in each specimen were evaluated in this experiment, it cannot be concluded that these do not harbor fetal cells. Therefore, a greater number of cells should be attempted analyzed.

A larger sample size is also beneficial for increased evidence, however contrary to the finding of a fetal cell amongst maternal cells, the frequency of women found to harbor FMC is often greater than 20% [105] [183] [184]. Further, analysis to unmask the characteristics of potential

fetal cells found may be conducted. Possibly, tissue specimens may be stained with different fluorescent cellular markers, such as markers for immune cells or endothelial cells, which may be applied simultaneously with FISH [185].

However, investigation of human tissues does come with limitations. The first being challenges of sample harvesting contributing to small sample sizes as already touched upon, not least regarding collecting healthy tissue samples as controls. The pregnancy history is also difficult to obtain, as has also been experienced with the donors of the atherosclerotic specimens used in this experiment. Therefore, animal studies prove their relevance. For example, mice can be genetically modified in such a way that the resulting fetal cells carry a fluorescent marker making it easy to distinguish fetal cells from maternal cells both during and after pregnancy using fluorescence microscopy [185]. The marking of fetal cells with a fluorescent marker also readily allows for cell sorting by flow cytometry whereby the cells are sorted in accordance with the amount of fluorescence they carry. This would allow for isolation of fluorescent fetal-origin cells from blood which could be used for further analysis [186]. Such analysis may be used for investigation of their differentiation potential for example. In humans, microchimeric cells can be separated from background cells by using fluorescent antibodies targeted at HLA-antigens, whereby one antibody is specific for the mother’s cells and one for the fetus's cells. Contrary to the labor-intensive challenge arising using HLA-specific probes in FISH, they identified 10 different HLA-antibodies which were calculated to cover over 90% of the HLA-mismatches between mothers and their offspring [187] [188].

Atherosclerosis and autoimmune disease both share the common feature of chronic inflammation [189]. Considering this, fetal microchimerism at varying frequencies has been found in the thyroids of women with Hashimoto Thyroiditis, an autoimmune disease attacking thyroid cells, compared to the findings of no FMC in the thyroids of healthy women [190] [98]. Furthermore, in Sjögren's syndrome (SS), an autoimmune disease attacking the exocrine glands, 36% of diseased labial salivary gland samples were positive for fetal microchimerism, whereas healthy control tissues showed none. Further, when examining the bronchoalveolar lavage fluid from women with SS compared to a control group without SS but with other lung diseases, fetal microchimerism was detected at a percentage of 22% and 0% respectively. In the same study, the prevalence of microchimerism in the women with SS was significantly higher in the diseased tissue samples than in the peripheral blood from the same women, in which no microchimerism was found [131].

The role of fetal microchimerism in autoimmune diseases remains unclear, one hypothesis being that fetus-derived immune cells directly attack tissues of the host thereby giving a situation like graft versus host disease in case of tissue transplantation, in which the graft refers to tissue donated and host to the tissue of the recipient. The complication arises when immunocompetent cells of the graft recognize the host's cells as foreign. Following this, an attack is initiated leading to inflammation, damage and scarring of host-tissue, thereby giving symptoms like that found in autoimmune diseases [191-193]. Patients with graft versus host disease are also found to have increased intima thickening of the carotid artery, an early sign of atherosclerosis [194]. If indeed fetal cells play a role in autoimmune disease, there are two circulating hypotheses. Either the fetal cells themselves differentiate into immune cells reacting to the mother's tissue like that in graft versus host disease, or the mother's immune system recognizes the fetal cells as partially foreign in which an immune response is evoked.

Considering the proposed allo-immune effects of FMC in autoimmune diseases, fetal cells may contribute to atherosclerosis in an allo-immune manner by detecting components of the plaque as "non-self" and thereby initiating inflammation. On the contrary, the fetal cells may be present in the vessel tissue and be detected as foreign by the maternal immune system. Regarding the former, autoimmune reactions are usually due to the mechanism of eliminating immune cells reacting to "self" being defective, in which they will wrongly attack self-tissue. This is a process in the maturation of immune cells and if the fetal cells are transferred into the mother as stem cells, the differentiation and maturation into immune cells will take place in the surroundings of maternal components, i.e., cells that react to maternal antigens will also be eliminated [195]. The widely accepted thought of fetal cells being transferred with a stem cell like character is supported by the findings of differentiated fetal cells in several maternal tissues, such as liver cells and epithelial cells [94].

Furthermore, if fetal cells induced autoimmune disease like what is thought in graft versus host disease, with the graft referring to the fetus and the host to the mother, the similarities between them would possibly be of a greater uncanniness, however they have been shown to differ in the symptomatic picture and pathogenesis For example, in Sjögrens syndrome antibodies reacting to ribonuclear proteins were found to be involved in the diseased exocrine glands in graft versus host disease. Further, the nail-fold of patients with systemic sclerosis show enlarged capillaries, microhemorrhages and avascular areas. None of which is found in patients with graft versus host disease. [196, 197].

If the fetal cell itself was to serve the function of the antigen for the mother's immune system to react to, it may have engrafted itself into healthy tissue and thereafter induced an immune response. This may be contradicted by the findings of FMC in the blood and tissues of healthy women [90, 198, 199]. Moreover, in atherosclerosis, contrary to autoimmune disease, the initial inflammatory reaction is not *as* much of an enigma. The accumulated oxidized LDL, for example, is one particle exhibiting pro-inflammatory properties and the initial inflammation in atherosclerosis is possibly more a response to abnormal tissue changes in the vascular wall than an attack on healthy tissue [42].

Further, fetal cells have been found in non-autoimmune diseased tissue from women with the viral infection hepatitis C, affecting the liver [93] and, in breast cancer patients, fetal cells were found in 21% of the women compared to 56% in the healthy control group, implying a protective effect [200]. Also, in lung samples from post-reproductive women with lung cancer, male cells were found to be clustered around the diseased tissue rather than in adjacent healthy areas, again speculated to be of stem cell origin [201]. Moreover, the finding of fetal cells in the caesarean scars of women who had undergone a c-section sparked the idea of fetal microchimerism in maternal wound healing. Together with previous findings of fetal cells in the bone marrow [105] it is proposed that fetal cells engraft in the bone marrow and are recruited along with maternal cells upon skin injury [104]. These findings taken together may steer FMC's role away from an inflammatory initiator to rather having a positive effect in resolving tissue damage. Like the fetal cells found in the c-section, the hepatitis-C liver specimens, and lung tissue, fetal cells in atherosclerosis may be recruited along with other immune cells from the bone marrow. This may also explain their presence in autoimmune-diseased tissue, but as the part of the general inflammatory response rather than the trigger.

Regarding cell type, in a study looking at the cellular phenotype of fetal cells in various diseased tissues, most were found to have fetal cells expressing CD45, a common leukocyte marker whilst simultaneously having seemingly identical morphology to the surrounding maternal cells. This suggests that although differentiated into tissue-specific cells, they originally derive from blood cells, further supporting the hypothesis that the cells are recruited upon tissue damage whereupon they graft into, and differentiate according to, the local tissue [94]. In the same study they also stained for cytokeratin in which fetal cells were found positive for in epithelial tissues, however no cells were found positive for both CD45 and cytokeratin indicating that there may also be a fetal epithelial progenitor cell.

Fetal microchimerism's role in tissue repair may also fit in the finding of increased fetal microchimerism in pregnant women with preeclampsia compared to healthy controls. In the study, the cells were found in the PBMC compartment of the blood. However, it was concluded unknown whether the cells were intact fetal cells or nucleated syncytial aggregates. Preeclampsia's pathology on tissue-level can be characterized by endothelial lesions in various organs [202]. Based on findings of fetal cells in appendix-specimens of women who had undergone appendectomy during pregnancy, and in greater numbers in more inflamed areas, this supports the role of fetal cells in tissue repair during pregnancy [171]. Likewise, more fetal cells may thus be recruited upon the event of preeclampsia, attempting to combat the resulting tissue-damage. Fetal cells' beneficial role in tissue repair might be due to their ability to heal through regeneration rather than the formation of scar tissue, in addition to being more rapid and with lesser inflammation [203]. However, as one proposed etiology of preeclampsia is poor maternal-fetal tolerance, a possibility may be that fetal cells derived from pregnancies complicated by preeclampsia are more prone to activate maternal immune cells long-term than fetal cells derived from pregnancies characterized by higher maternal tolerance. This would be in line with the increased epidemiological risk of CVD in women with a history of preeclampsia [6].

A question which remains unanswered is the true cellular origin of fetal cells within the maternal body. Khosrotehrani and Bianch argue that it is difficult to imagine fully differentiated fetal cells with no capacity of self-renewal having the ability to persist in the mother's body for decades, as has been shown. It therefore seems reasonable to assume they are of a stem-cell character, which is a widely accepted hypothesis. The question then evolves to where those stem cells arise from, the main two candidates being mesenchymal stem cells or hematopoietic stem cells, or even possibly a novel unknown stem cell type. The hematopoietic stem cell gives rise to all cells of the blood, including the immune cells [204]. The definition of a mesenchymal cell is not as clear-cut; however, they are shown to give rise to bone cells, cartilage cells, fat cells and heart- cells, amongst others. Further, when mesenchymal cells are infused in patients, the cells are found to engraft in tissues and have been shown to be stable long term and even to migrate specifically to sites of injury [205]. This is possibly similar if mesenchymal cells are transferred from the fetus to maternal circulation. As trophoblasts are the only cells of the placenta in direct contact with maternal blood and found in maternal circulation [206], another hypothesis may be that these are the true origin of fetal microchimerism. However, as these are differentiated trophoblasts, they would need to undergo a cell-lineage switch to provide the

cells found in various maternal tissues. Indeed, it has been shown that trophoblasts from mice can be reversed back to pluripotent cells by overexpressing certain genes in vitro, which then would have the capacity to differentiate into cells of any tissue [207].

Taken together, many questions regarding the origin, nature, and effect of microchimerism remain. Confirming the possible finding of an XY-positive cell within female atherosclerosis, using the measures discussed above, could have big implications for our understanding of female atherosclerosis pathophysiology. In addition, while fetal microchimerism is a phenomenon exclusive to women, maternal microchimerism is likely universal, prompting further research into the origin of the cells within male atherosclerosis as well.

7 Conclusions

1. In this project, a ddPCR protocol for detecting fetal microchimerism was established. However, due to the previously demonstrated low rate of fetal cells in maternal blood and tissue, the amount of DNA yielded from our preceding DNA extraction from atherosclerotic tissue was not sufficient to ensure detection of potentially present fetal cells. Therefore, the method of DNA isolation needs to be altered to increase DNA yield, either through a different method completely or by modifying certain steps.

2. FISH for the detection of male cells in female tissues was successfully established for placenta and decidua basalis. However, further optimization is still needed for the detection of male cells in atherosclerotic tissue. The results among and within atherosclerotic tissues were not uniform, with some regions and tissues showing weak signals and a lot of background. It remains uncertain if this is something that may be improved by modifying the protocol further or if the plaque tissue is not well suited for FISH, either due to tissue type or storage conditions such as fixation time; the former supported by the successfulness of FISH performed on placenta and decidua. However, for the most part, FISH results were sufficient for analysis of probe-signals. Alternative methods of analyzation must also be considered.

3. A cell positive for both the X and Y chromosome was detected in one atherosclerotic sample using FISH. As atherosclerosis is amongst others characterized by dysfunctional endothelium and inflammation, this may support our hypothesis that fetal cells engrafted in the endothelium may act as an inflammatory trigger through their allogeneic character. Alternatively, presence of FMC in maternal endothelium could also reflect a potential role of FMC in tissue repair, trying to combat and resolve the chronic inflammation. Information about what kind of cells home to atherosclerotic tissue and their location may be needed to say more about the reason for their presence. One way of reaching such goals would be to stain the tissue with fluorescent cellular markers, which can be applied simultaneously with FISH.

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