Energy metabolism and angiogenesis in atherosclerosis and cancer

Matias Ekstrand

Department of Molecular and Clinical Medicine

Institute of Medicine

Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

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Cover illustration: Intussusceptive Angiogenesis by Matias Ekstrand

For science

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Department of Molecular and Clinical Medicine, Institute of Medicine
Sahlgrenska Academy, University of Gothenburg
Gothenburg, Sweden

ABSTRACT

Energy metabolism requires supply of glucose and oxygen. In atherosclerotic plaques and cancer tumors, there are local areas with mismatch in demand and supply of oxygen and nutrients. The resulting cellular energy imbalance may promote energy failure and stimulation of angiogenesis.

In the first paper, energy metabolites were analyzed in human atherosclerotic plaques using high-resolution bioluminescence imaging. Advanced plaques were deficient in ATP and glucose, whereas lactate accumulated. ATP and glucose deficiency was most pronounced in macrophage-rich areas adjacent to the necrotic core. ATP depletion may promote necrotic core expansion and progression from stable to unstable plaques.

In the second paper, reactive oxygen species (ROS) production was studied during the development of atherosclerosis in mice. Intracellular ROS levels increased before lesions were visible, suggesting that intracellular ROS promote initiation of atherosclerosis. In advanced atherosclerotic plaques, atorvastatin decreased ROS production in a lipid-lowering independent manner. The decrease in ROS may promote stabilization of plaques.

In the third paper, intussusceptive angiogenesis (IA) was demonstrated in human, but not mouse, melanoma metastases. IA may contribute to the growth of human melanoma metastases and help explain the poor effect of current anti-angiogenic drugs targeted to classic sprouting angiogenesis. We further demonstrated that MMP inhibition blocks IA *in vitro*.

In summary, this thesis provides evidence of energy deficiency in human atherosclerotic plaques, new insights into ROS distribution during atherosclerosis development, and finally, evidence of intussusceptive angiogenesis in human malignant melanoma metastases. These data may be used to further the research into better treatments of atherosclerosis and cancer.

Sammanfattning på svenska

Energimetabolism på cellnivå är beroende av tillgången på syrgas och socker. När det blir brist på syrgas (hypoxi) eller socker resulterar det i ett antal effekter, varav några är produktion av fria syreradikaler (ROS), och kompensatorisk blodkärlsnybildning (angiogenes) för att återställa energitillgången. I denna avhandling har jag studerat olika effekter av syrebrist och energibrist i ateroskleros och cancer.

I den första artikeln analyserades mänskliga aterosklerotiska plack (åderförkalkning) avseende energimetaboliter. Genom att använda metoden bioluminiscens imaging konstaterades att avancerade plack har brist på ATP och socker, vilka behövs för att celler ska överleva. Dessutom noterades att laktat ansamlades i avancerade plack.

I den andra artikeln analyserades närvaron av ROS i levande mus-aorta under utvecklingen av ateroskleros. Genom att använda bioluminiscens och ROS-prober påvisades en ökning av ROS innan synliga plack hade utvecklats. Dessutom minskade läkemedlet atorvastatin nivåerna av både intracellulärt och extracellulärt ROS aterosklerotiska plack, oberoende av lipidnivåer.

I den tredje artikeln studerades en typ av blodkärlsnybildning kallad intussusceptiv angiogenes (eller splitting-angiogenes) i malignt melanom hos patienter. Genom att använda immunofluoroscens visade vi att intussusceptiv angiogenes förekommer i malignt melanom hos människa, men inte i två musmodeller av malignt melanom. Att intussusceptiv angiogenes förekommer i mänsklig cancer kan vara en anledning till att nuvarande cancerbehandlingar som riktar in sig på blodkärlsnybildning inte är effektiva i de flesta cancertyper.

Sammanfattningsvis kan sägas att min avhanding har producerat belägg för energibrist i avancerade mänskliga aterosklerotiska plack, att intracellulärt ROS ökar innan utveckling av ateroskleros, samt förekomsten av splitting-angiogenes i humant malignt melanom. Dessa data kan i samband med framtida forskning förbättra behandlingen av ateroskleros och malignt melanom.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Depletion of ATP and glucose in advanced human atherosclerotic plaques**

   Ekstrand M, Widell E, Hammar A, Akyürek LM, Johansson M, Fagerberg B, Bergström G, Levin MC, Fogelstrand P, Borén J, Levin M.

   PLoS One. 2017 Jun 1;12(6):e0178877. PMID: 28570702

II. **Imaging of Intracellular and Extracellular ROS Levels in Atherosclerotic Mouse Aortas Ex Vivo: Effects of Lipid Lowering by Diet or Atorvastatin.**

   Ekstrand M, Gustafsson Trajkovska M, Perman-Sundelin J, Fogelstrand P, Adiels M, Johansson M, Mattsson-Hultén L, Borén J, Levin M.

   PLoS One. 2015 Jun 22;10(6):e0130898. PMID: 26098110

III. **Intussusceptive angiogenesis in malignant melanoma**

   Ekstrand M, Pandita A, Bjursten S, Ekelund E, Fogelstrand P, Le Gal K, Nilsson J, Ny L, Johansson I, Bergö M, Akyurek LM, Levin MC, Boren J, Ewald AJ, Mostov K, Levin M.

   Manuscript

CONTENT

ABBREVIATIONS

ADP/ATP	Adenosine diphosphate / Adenosine triphosphate
BMDC	Bone marrow-derived cell
BPT	*Braf$^{CA/+}$Pten$^{f/f}$Tyrosinase-Cre* (mouse model)
CoA	Coenzyme A
DNA	Deoxyribonucleic acid
ER	Endoplasmatic reticulum
GDP/GTP	Guanosine diphosphate / Guanosine triphosphate
HIF	Hypoxia-inducible factor
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
MMP	Matrix metalloproteinase
NAD$^+$/NADH	Nicotinamide adenine dinucleotide (oxidized/reduced)
NO	Nitric oxide
NOS	Nitric oxide synthase
Nox	NADPH oxidase
PDX	Patient-derived xenograft (mouse model)
ROS	Reactive oxygen species
SOD	Superoxide dismutase
VEGF	Vascular endothelial growth factor

DEFINITIONS IN SHORT

Normoxia — Normal oxygen level. Sometimes refers to atmospheric oxygen concentration (21 %). Occasionally refers to physoxia.

Physoxia — Physiological oxygen level. The normal oxygen level in a certain tissue.

Hypoxia — Oxygen level below physoxia. A lack of oxygen.

1 INTRODUCTION

1.1 OXYGEN IN EVOLUTION

Earth is estimated to be 4.5 billion years old, and life on earth is considered to have emerged around 3.7 billion years ago [1]. The conditions of early life were entirely anoxic, meaning no oxygen was present, and metabolism taking place under these conditions is said to be anaerobic.

2.3 billion years ago, cyanobacteria started producing oxygen as a byproduct of photosynthesis. This oxygen was initially captured by dissolved iron in the oceans, and oxygen levels remained low. The oxygen-capture system by iron became saturated about 2.25 billion years ago, and the levels of oxygen in the oceans and atmosphere started rising in what is called "the great oxygenation event". Even though atmospheric oxygen was a new occurrence, aerobic respiration on a limited scale was thought to have previously existed in environments with water photolysis [2], or through catalase enzymes which convert hydrogen peroxide to oxygen and water [3].

Most of the life present during the great oxygenation event would today be classified as obligate anaerobes, to which oxygen is toxic. Cells needed to adapt to this change in oxygen levels by avoiding it altogether, by developing systems to tolerate oxygen, or even using oxygen to extract energy.

Early eukaryotic cells, which separated their DNA from the rest of the cell by using a nuclear membrane, were initially adapted to using only anaerobic metabolism [4]. According to the endosymbiosis theory, early eukaryotes engulfed a bacterium, a proto-mitochondrion, which got enclosed in a membrane in a similar way the DNA of the nucleus was enclosed [5]. A symbiosis developed where the bacterium, eventually called mitochondrion, utilized oxygen for energy production through respiration, while the host eukaryotic cell provided most of the remaining metabolic functions. Over time, some of the mitochondrial DNA moved to the cell nucleus, and a translocation system for directing proteins to the mitochondrial compartment evolved [6]. Even today, the mitochondria keep some of their bacterial features, such as unique protein synthesis and circular DNA, and binary fission, which is dissimilar to the rest of the eukaryotic cell. Through this symbiosis between the mitochondria and early eukaryotic cell, eukaryotes managed to adapt to the increased levels of oxygen in the atmosphere.

There are numerous advantages of aerobic metabolism. One such advantage is that aerobic metabolism produces considerably more energy than anaerobic metabolism. In addition to higher energy production, the presence of oxygen enabled more than 1000 new metabolic reactions to develop [7]. In an aerobic environment, transmembrane proteins can contain oxygen-rich external domains vital for cellular signaling, which in turn is required for multicellular life [8].

Unicellular organisms and smaller multicellular organisms receive oxygen by passive diffusion over their cell membranes. As a multicellular organism grows, oxygen is gradually consumed by the outermost cells, which creates an oxygen gradient. This oxygen gradient causes the innermost cells to experience a lack of oxygen, which in turn results in metabolic issues or cell death. That oxygen has a diffusion limit of approximately 200 μm creates a size limit of multicellular life unless transport of oxygen is facilitated in some manner. Humans are equipped with a closed circulatory system where blood is transported through blood vessels powered by the heart, and gas exchange takes place in our lungs [9].

For oxygen-dependent organisms, hypoxia causes a variety of issues: their metabolism cannot function properly, movement is impaired, energy-consuming processes are halted, damaging reactive oxygen species are released, and the cell may ultimately die unless oxygen levels are restored.

Cells have pre-programmed responses to prevent cellular damage and death when they sense hypoxia [10]. They release a variety of signals to induce increased oxygen and energy supply, as well as reducing energy-demanding processes and changing their energy production to function in an anaerobic environment. However, these responses sometimes go awry, and the response designed to correct an energy imbalance instead worsens the energy imbalance or promotes disease progression.

1.2   ENERGY PRODUCTION WITH AND WITHOUT OXYGEN

The main purpose of energy metabolism is to produce ATP (adenosine triphosphate), which is a universal energy carrier of the cell. When ATP is used to drive various cellular processes, one or two phosphate groups are cleaved from the molecule, which releases the stored chemical energy. After ATP is consumed, ADP (adenosine diphosphate) or AMP (adenosine monophosphate) remains, along with free phosphate groups.

Glucose is an energy carrier that may easily be moved between cells within an organism and can be stored polymerized as glycogen. In all the upcoming descriptions of energy metabolism, we will use glucose as the starting point, even though many other energy substrates, such as amino acids and fatty acids, can be quickly metabolized and inserted into various parts of the metabolic pathways.

1.2.1   GLYCOLYSIS

All eukaryotes are equipped with a metabolic system called glycolysis [11], where one glucose molecule is broken down into two molecules of pyruvate. Glycolysis generates 2 ATP molecules from the original glucose molecule, or 3 ATP if the source is glycogen [12]. Compared to respiration, this is a very small energy gain; however, the flux through the glycolytic pathway is very rapid, meaning that energy can quickly be mobilized, given an adequate supply of glucose or glycogen.

Glycolysis takes place in the cell cytoplasm, and the process is anaerobic, i.e., it does not require oxygen. In addition to creating pyruvate, NAD^+ (nicotinamide adenine dinucleotide) is reduced to NADH during glycolysis. The available pool of NAD^+ in the cytoplasm is fairly small, which means it needs to be restored if glycolysis is taking place rapidly. NAD^+ can be restored anaerobically by conversion of pyruvate into lactate by lactate dehydrogenase (LDH). Alternatively, the electrons of NADH can be transferred into the mitochondria by the help of the malate-aspartate shuttle, after which mitochondrial NAD^+ can be restored by oxidative phosphorylation in the presence of oxygen, which results in the production of ATP.

After pyruvate is produced through glycolysis, it is transported into the mitochondrial matrix, where it is oxidized into acetyl-CoA (acetyl coenzyme A).
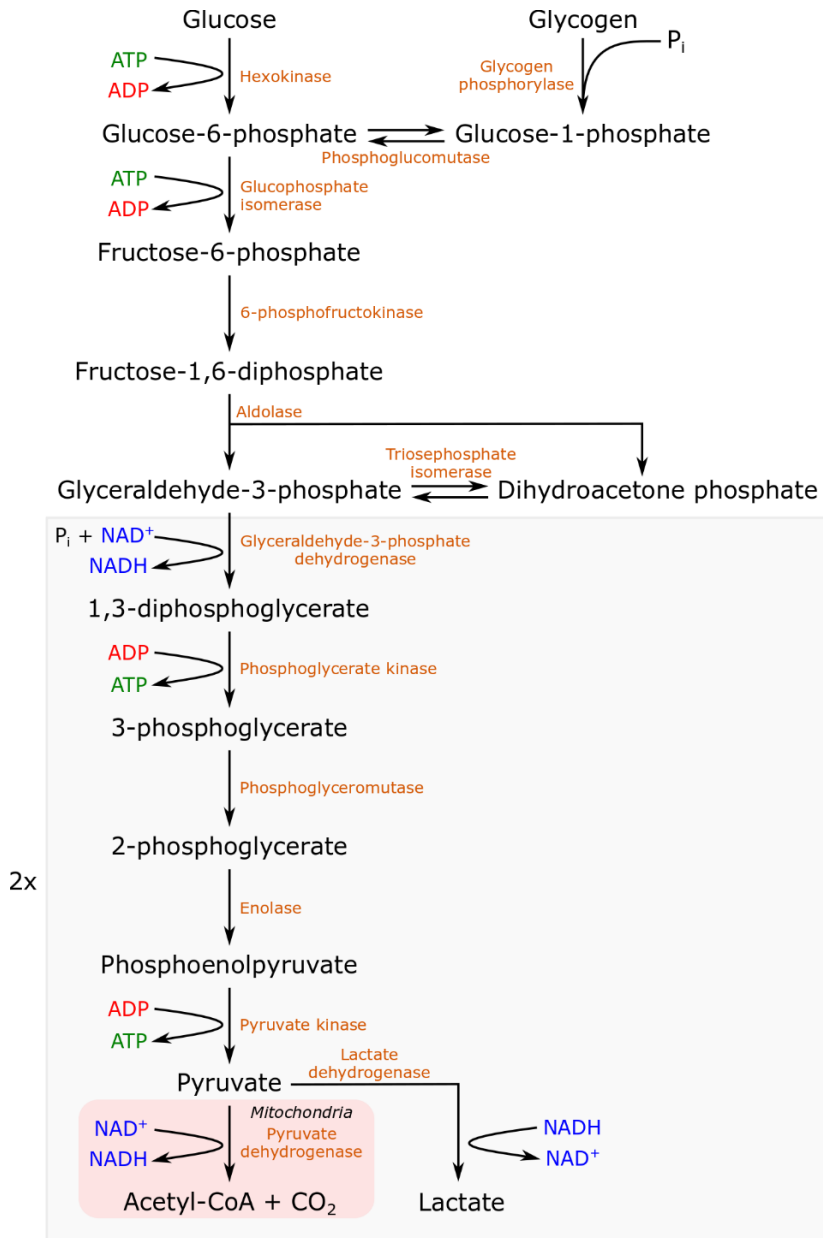
Figure 1 - Glycolysis pathway. Glucose or glycogen is metabolized into Acetyl-CoA or lactate while producing 2-3 ATP depending on the glucose source. Orange text signifies enzymes. Red text signifies low energy compounds and green text signifies high energy compounds. Blue text signifies redox pairs. All content inside the gray box takes place twice per original glucose molecule. All reactions occur in the cytosol unless specified otherwise.

1.2.2   CITRIC ACID CYCLE

Acetyl-CoA enters the citric acid cycle (Figure 2) with the aid of citrate synthase, which converts it into citrate. Once in the cycle, the citrate is metabolized by a series of enzymes which create a variety of compounds along the way. Several steps in the citric acid cycle create NADH, which is later used in oxidative phosphorylation. The reaction of succinate into fumarate takes place with the aid of complex II, which also reduces ubiquinone (Q) to ubiquinol (QH_2). This process requires functional oxidative phosphorylation, and without it, the citric acid cycle will cease to regenerate the substances needed to continue the cycle.
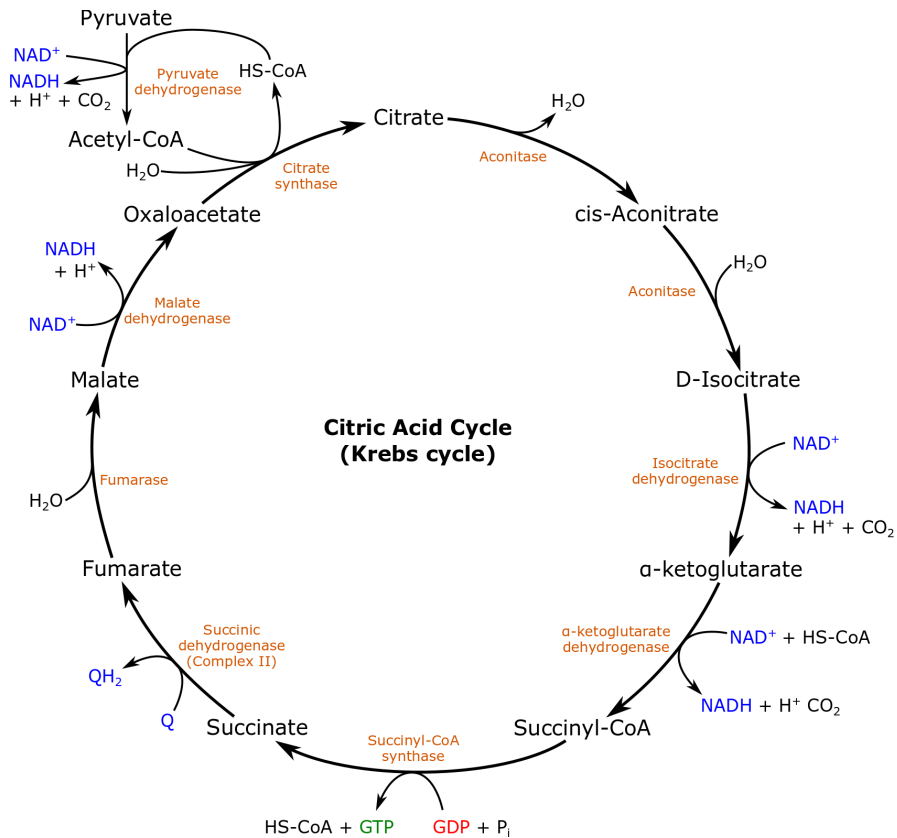
Figure 2 - In the citric acid cycle, the acetyl part of acetyl-CoA is attached to oxaloacetate to generate citric acid. Through a series of reactions, the two extra carbons are oxidized into CO_2, recreating citrate, while also producing NADH from NAD^+, as well as other energy-containing compounds. NADH and QH_2 can further be processed through oxidative phosphorylation. Orange text signifies enzymes. Red text signifies low energy compounds and green text signifies high energy compounds. Blue text signifies redox pairs.

1.2.3   OXIDATIVE PHOSPHORYLATION

Once NADH is produced in the citric acid cycle, it reacts with complex I of the electron transport chain to release two electrons, which are deposited on Q to form QH_2 while oxidizing NADH to NAD^+ and H^+. The electrons of QH_2 are passed between complex III and IV, which both use the energy of the electrons to drive an H^+ pump, transporting H^+ from the matrix into the intermembrane space of the mitochondria. In the end, the electrons are deposited on oxygen (the terminal electron acceptor), which produces water as the end product.

In addition to using NADH as a source of electrons, succinate, which is part of the citric acid cycle, is also used as an electron source for the electron transport chain. Succinate reacts with complex II, which oxidizes succinate to fumarate, and passes the electrons to Q to produce QH_2, whose electrons are shuttled through complex III and IV, and pumps H^+ from the matrix into the intermembrane space.

The H^+ gradient which is built up by the electron transport chain can then be utilized by ATP synthase, allowing H^+ back into the matrix in a controlled manner while facilitating the reaction of ADP and a phosphate group, producing ATP [13]. When the entire pathway from glucose into carbon dioxide and water is taken into account, oxidative phosphorylation produces approximately 30-38 molecules of ATP [14, 15] from each molecule of glucose.
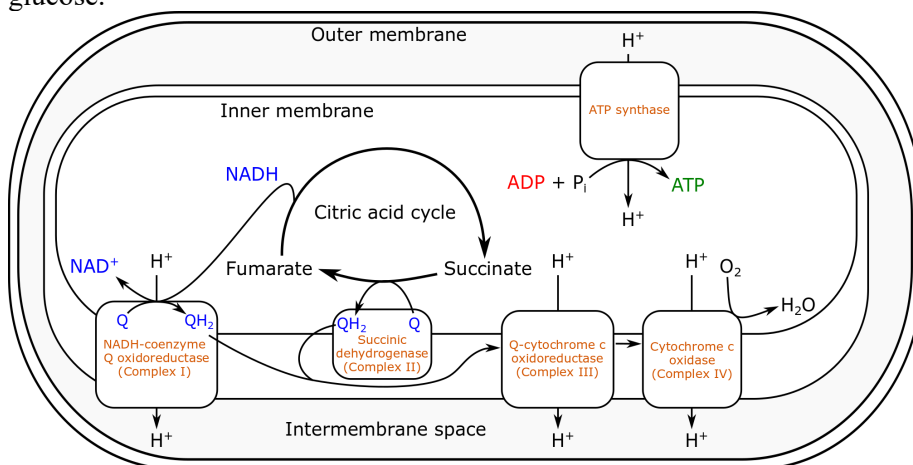
Figure 3 - Oxidative phosphorylation. Electrons from NADH are transferred to QH_2 in complex I accompanied by the transport of H^+ into the intermembrane space. Concurrently, succinate is converted to fumarate in complex II, also producing QH_2. The electrons of QH_2 are passed though complex III and IV accompanied by the transport of H^+ into the intermembrane space, finally reducing oxygen to produce water. The proton gradient is used by ATP synthase to produce ATP. Orange text signifies enzymes. Red text signifies low energy compounds and green text signifies high energy compounds. Blue text signifies redox pairs.

1.2.4 OTHER MEANS OF ENERGY PRODUCTION

The pathways mentioned so far (i.e., glycolysis, the citric acid cycle, and oxidative phosphorylation), are important in energy metabolism. However, there are also other metabolic pathways which can be utilized to generate energy.

Fatty acids can be broken down by a process called beta-oxidation. In short, free fatty acids are transported to the mitochondrial matrix by the aid of membrane transport proteins, after having been conjugated to CoA, to form fatty acyl-CoA. Once inside the mitochondrial matrix, beta-oxidation takes place, where carbon atoms are cleaved in pairs from the fatty acid chain to form acetyl-CoA until the entire chain has been consumed [16]. Acetyl-CoA can then be fed into the citric acid cycle and processed from there following previously mentioned pathways. Each cycle of beta-oxidation produces approximately 14 ATP and is dependent on oxidative phosphorylation to function.

Amino acids can de deaminated, where the amino group is removed from the rest of the carbon skeleton. If the remaining part of the amino acid is glucogenic, it can be metabolized into citric acid cycle intermediates, which may be broken down for energy or go through gluconeogenesis. If the amino acid is ketogenic, it may be converted to acetyl-CoA, which in turn can generate energy, or be used in ketogenesis to produce ketone bodies [17].

During periods of low food intake, fasting, or starvation, the liver may create ketone bodies to supply the body with energy [18]. Mainly muscle, heart, and brain tissue will use ketone bodies as an energy source through re-conversion into acetyl-CoA, which is metabolized through the citric acid cycle and downstream pathways to produce energy.

1.2.5 OXYGEN REQUIREMENTS FOR ENERGY PRODUCTION

Oxidative phosphorylation is the major component of human energy metabolism which requires oxygen. Most of our energy metabolism is reliant on oxidative phosphorylation to restore redox balance and produce ATP. Without oxidative phosphorylation, the citric acid cycle cannot restore fumarate from succinate, and other reduced species such as NADH start to accumulate. Glycolysis is the only truly anaerobic pathway able to continuously generate energy in human cells, and it generates only 2-3 ATP per glucose molecule, compared to the 30-38 ATP that can be created through full respiration [14, 15].

1.3 INSUFFICIENT OXYGEN SUPPLY – HYPOXIA

In the atmosphere, the oxygen concentration is 21 %, which is called normoxia. The normal level of oxygen in a certain tissue is called physoxia, which is much lower than 21 %. Some examples of oxygen levels in different parts of the body are given in Table 1.

Table 1 - Oxygen concentrations in different organs and tissues. Values collected from Correau et al. [19].

Tissue	Physoxia level (% oxygen)
Atmosphere	21.1 %
Air in the alveoli	14.5 %
Arterial blood	13.2 %
Venous blood	5.3 %
Cell	1.3-2.5 %

When oxygen levels are much lower than normal for a certain tissue, it is called hypoxia, the deficiency of oxygen. Cells have a pre-programmed response to hypoxia to adapt and survive, and it usually starts to occur when oxygen levels reach around 5% and gradually increases as oxygen levels fall [20]. Currently, hypoxia is defined as an oxygen concentration of less than 2% [21].

Hypoxia is present in a variety of physiological as well as pathological conditions. Embryo development, for example, is entirely dependent on functional hypoxic signaling to survive [21, 22]. Hypoxia is also required to keep stem cells in an undifferentiated state [23]. Local tissue hypoxia is also a feature of several pathologies, such as tumors [24], atherosclerotic plaques [25], and chronic wounds [26].

On a systemic level, acute hypoxia in the arterial blood is sensed by chemoreceptors in the carotid body, which induce hyperventilation to alleviate the lack of oxygen [27]. Chronic hypoxia is compensated by increased production of erythropoietin (EPO) in the kidney, stimulating the production

of red blood cells for more efficient extraction of oxygen from the air in the lungs, and an increased oxygen transport capacity [28].

When hypoxic stress is detected by the cell, the hypoxic response is triggered. In mammals, this is mediated mainly by hypoxia-inducible factors (HIFs), which are transcription factors that alter the expression of more than 100 genes [29]. The overall purpose of the hypoxic response is to promote survival of the cell by downregulating energy-demanding processes, upregulating anaerobic metabolism, and promoting energy restoration to the tissue in which the cell resides.

1.3.1   HYPOXIA INDUCIBLE FACTORS

HIF, a heterodimer, is a protein consisting of two different subunits, HIFα and HIFβ. HIFα is oxygen labile, meaning it breaks down in the presence of oxygen, while HIFβ (also called ARNT) is stable and continuously expressed regardless of the oxygen level.

Table 2 - HIF consists of an alpha and beta subunit. The alpha subunit exists in several isoforms and is oxygen labile, while the beta subunit is oxygen stable and continuously expressed.

	HIFα +	HIFβ
HIF-1	HIF1α	HIFβ
HIF-2	HIF2α	HIFβ
HIF-3	HIF3α	HIFβ

HIFs bind to hypoxia response elements (HRE) on DNA to convey their transcriptional effects. While HIF-1 and HIF-2 bind to the same HRE, their target genes are slightly different [30]. HIF-3, on the other hand, negatively regulates HIF1 and HIF-2 either by direct competition or by disrupting the interaction of HIFα and HIFβ subunits [31].

The effects of HIF-1 generally include a shift toward anaerobic glycolysis by upregulation of glycolytic enzymes, such as hexokinase, phosphofructokinase, aldolase, and many others. HIF-1 also upregulates glucose transporters (GLUTs) and prevents the entry of pyruvate into the citric acid cycle by upregulation of pyruvate dehydrogenase kinase 1 (PDK1), which stops the conversion of pyruvate into acetyl-CoA by phosphorylating pyruvate dehydrogenase (PDH) [32]. Additionally, LDH is upregulated, which

promotes the conversion of pyruvate into lactate while restoring NAD^+ to allow further glycolysis.

The effects of HIF increase cellular import of glucose, flux through the glycolytic pathway and finally conversion into lactate. Since the flow of metabolites into the citric acid cycle is prevented, the need for oxidative phosphorylation, and subsequently, the need for oxygen is reduced.

Not only does HIF signaling promote immediate survival through metabolic redirection for energy production, but also redirects parts of the glucose metabolism into the pentose phosphate pathway (PPP) to be used for DNA and RNA synthesis, as well as the production of NADPH which is used to restore antioxidant function [33].

The effects mentioned so far have been centered around the cellular adaptation to hypoxia. Ideally, hypoxia needs to be resolved, and this is where the cell needs to affect its surroundings. HIF signaling promotes the production of vascular endothelial growth factor (VEGF) [29] which induces angiogenesis, the formation of new blood vessels, to restore oxygen supply and resolve the hypoxic stress on the cell.

HIF signaling is tightly connected to inflammation. In most inflammatory conditions, cells experience hypoxia due to lower circulation, and high metabolic demand of infiltrating immune cells. During inflammation, nuclear factor kappa B (NF-κB) is activated through a separate pathway, as well as by HIF. Interestingly, NF-κB also upregulates HIF1α the transcription in macrophages [34].

1.3.2   REGULATION OF HIF

When oxygen is present, prolyl hydroxylase domain (PHD) proteins hydroxylate HIFα at two conserved proline residues [35]. This hydroxylation makes an E3 ubiquitin ligase identify HIFα as marked for proteasomal degradation, meaning HIFα is degraded in the presence of oxygen [36].

PHDs, in turn, are directly regulated by oxygen. PHDs require α-ketoglutarate as a substrate, which is an intermediate in the citric acid cycle. Other intermediates of the citric acid cycle, such as fumarate and succinate, instead inhibit PHDs, which allows for fine-tuning of the pathway depending on metabolic status. Additionally, during moderate hypoxic conditions of approximately 1.5 % oxygen, mitochondria produce reactive oxygen species (ROS) from mitochondrial complex III, which then inhibit PHD activity, and thus stabilize HIFα [37].

Factor-inhibiting HIF1α (FIH1) is another oxygen-dependent enzyme which hydroxylates HIF1α at an asparagine residue, which in turn prevents HIF1α from binding to its coactivators [38, 39]. There are other regulators of HIF as well, such as sirtuins, which modulate HIF depending on the cellular redox state [40].

1.3.3   OTHER OXYGEN SENSING PATHWAYS

While HIFs are the most known oxygen-sensing pathway, there are also other pathways that cooperate to induce hypoxia-tolerance. These include the kinase mammalian target of rapamycin (mTOR), the unfolded protein response (UPR), and NF-κB [41, 42].

1.4 REACTIVE OXYGEN SPECIES

Reactive oxygen species (ROS) are reactive oxygen-containing compounds, which are created as a byproduct of cellular metabolism or intentionally formed as part of signaling pathways or cellular defense.

The existence of ROS in biology has been known for approximately 60 years [43]. Initially, the presence of ROS was regarded as an exclusively deleterious process, leading to oxidative damage, cancer, and aging. However, that view was later revised, as ROS were shown to be part of normal physiological processes and signaling [44].

There are many types of ROS. Some examples are superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^\bullet OH$), singlet oxygen ($O_2^\bullet$) and peroxynitrite ($ONOO^-$) [44]. Each type of ROS functions slightly differently in terms of location, reactivity, as well as signaling pathways and diseases they are involved in.

1.4.1 REACTIVE OXYGEN SPECIES IN PHYSIOLOGICAL CELL SIGNALING

Production of extracellular ROS is part of the pathogen defense and other signaling functions. NADPH oxidases (Nox) create superoxide or hydrogen peroxide aimed toward the extracellular space or intracellular phagosomes. Nox is expressed in 5 isoforms in a variety of cell types. Nox1 is expressed in epithelial cells of the colon (wound healing), Nox2 is expressed in macrophages and neutrophils (pathogen defense) [44], Nox3 is expressed in the inner ear (otolith production), Nox4 is expressed in kidney (stem cell maintenance), and Nox5 is expressed in testis (sperm maturation) [45].

Furthermore, nitric oxide synthase (NOS) is present in endothelial cells, generating nitric oxide, which regulates the vascular tone of smooth muscle around the vessel. Xanthine synthase is a hepatic enzyme which is released into the blood, converting hypoxanthine into xanthine and finally, uric acid while also produces superoxide and hydrogen peroxide [46].

Intracellular ROS is produced mainly by mitochondria. Both mitochondrial complexes I and III produce both baseline ROS, as well as increased ROS to direct intracellular signaling [47]. Intracellular ROS affect enzymes in various signaling pathways, such as ASK1 and PI3K (proliferation, survival signaling) [48, 49], IRP (iron homeostasis) [50], ATM (DNA damage repair and autophagy) [51], and Ref-1 (antioxidant response) [52]. ROS is also involved

in mitochondrial hypoxia signaling, as seen in chapter 1.3.2 "Regulation of HIF".

ROS exerts its physiological effects through an oxidative interface, described as redox-sensitive cysteine and methionine residues which ROS may interact with [53]. Depending on what degree the residues are oxidized to, the modifications are temporary (sulfenic acid, -SOH), hard to reverse (sulfinic acid, -SO$_2$H), or nearly irreversible (sulfonic acid, -SO$_3$H). The oxidative changes induce conformational changes of the proteins, as well as formation of intra-protein disulfide bridges in the case of sulfenic acid. The oxidative changes to cysteine and methionine residues on proteins can be reversed by thioredoxin, peroxiredoxin, and methionine sulfoxide reductase [54, 55].

1.4.2 NEUTRALIZATION OF REACTIVE OXYGEN SPECIES

The neutralization, or scavenging, of ROS is essential for protecting the cell against deleterious effects since an accumulation of ROS can damage intracellular and/or extracellular components.

Superoxide, one of the most potent types of ROS, is scavenged by superoxide dismutase (SOD) which converts superoxide into hydrogen peroxide. There are different isoforms of SOD, all of which have different localizations. SOD1 is cytoplasmic, SOD2 is mitochondrial, and SOD3 (also known as ecSOD) is extracellular [56].

Hydrogen peroxide, in turn, is converted into water and molecular oxygen by the enzyme catalase, which can be intracellular or extracellular [57]. In addition to neutralizing hydrogen peroxide, membrane-associated catalase also scavenges peroxynitrite and oxidizes nitric oxide [58].

Glutathione (GSH) is a soluble antioxidant, able to neutralize both hydrogen peroxide and lipid peroxides through intracellular or extracellular glutathione peroxidases (GPx) [59, 60]. After oxidation of glutathione, it may be reduced by NADPH to restore its anti-oxidative function [61].

1.4.3 EXCESSIVE ROS IN PATHOLOGICAL CONDITIONS

While a baseline of ROS is required for basic cellular functions, an excess of ROS results in potentially harmful effects. ROS has been shown to be connected to a variety of diseases, such as cardiovascular disease, autoimmune diseases, neurological disorders, and cancer [62].

An important aspect of ROS is its pro-inflammatory features. Extracellular ROS increases the adhesion of leukocytes to endothelium, meaning that immune cells are recruited to sites where ROS is present [63]. Moreover, intracellular ROS promotes an inflammatory macrophage phenotype [64]. This is especially important in diseases such as atherosclerosis, where high intracellular and extracellular ROS levels promote an ever-increasing inflammatory state [65].

1.5 TRANSPORT OF OXYGEN TO CELLS – BLOOD VESSELS

Unicellular organisms get access to oxygen through diffusion from the environment directly into the cell. Bigger multicellular organisms require a transport system for oxygen. In humans, this is accomplished by the vascular system. In the lungs, oxygen binds to hemoglobin in red blood cells which travel through the vascular system into the semi-permeable capillaries, where oxygen is released to the surrounding tissues. The vascular system is adapted so that capillaries are dispersed through the various tissues to provide the cells of the body with sufficient oxygen supply. Therefore, almost all cells in the body are situated within 100-200 μm from a capillary [66].

1.5.1 VASCULOGENESIS AND ANGIOGENESIS

The vascular system is created in two distinct phases – vasculogenesis and angiogenesis. Vasculogenesis is the *de novo* formation of blood vessels, where individual endothelial cells create a vessel. Vasculogenesis takes place in early development to establish the base of the vascular system of the growing embryo. In vasculogenesis, endothelial progenitor cells are recruited from the bone marrow to the tissue, where they form new vessels [67].

Once the initial embryonic vascular network is established, it is further expanded via angiogenesis. Angiogenesis is the formation of new blood vessels from existing vessels. There are two forms of angiogenesis, sprouting angiogenesis and intussusceptive (or splitting) angiogenesis. Sprouting angiogenesis is the outgrowth of a new branch from an existing vessel, and intussusceptive angiogenesis is the splitting of one vessel into two parallel vessels.

1.5.1.1 SPROUTING ANGIOGENESIS

Observations of what would later be called angiogenesis were first described around 1787 by a surgeon named John Hunter [68]. The angiogenesis observed was later termed sprouting angiogenesis. Sprouting angiogenesis has been the main focus of angiogenesis research, being the majority of the almost 100.000 scientific publications about angiogenesis that can currently be found.

Sprouting angiogenesis is dependent mainly on the signaling pathway of VEGF. When tissue requires better vascularization, VEGF is released by hypoxic cells. VEGF is sensed by VEGF receptors on endothelial cells of nearby vessels. When there exist enough stimuli to trigger sprouting, a tip cell is formed (Figure 4). The tip cell is described as having many filopodia (reminiscent of finger-like appendices), which sense angiogenic signals and

directs the growing sprout [69]. The tip cell is followed by stalk cells which are proliferative and phalanx cells which connect the sprout to the original vessel. The sprouts eventually fuse with other sprouts or blood vessels in the area, after which blood flow can be established inside the newly formed vessel.

There are a number of other factors which impact sprouting angiogenesis, such as fibroblast growth factors (FGF) [70], transforming growth factor β (TGF-β) [71] and platelet-derived growth factor (PDGF) [72, 73], matrix metalloproteinases (MMPs) [74], and many others [75, 76]. Many of these factors are directly or indirectly connected to VEGF signaling pathways.

Sprouting

Intussusception

**Angiogenesis
progression**

Tip cell

Pillar

Smooth muscle cell

Endothelial cell

Smooth muscle cell

Endothelial cell
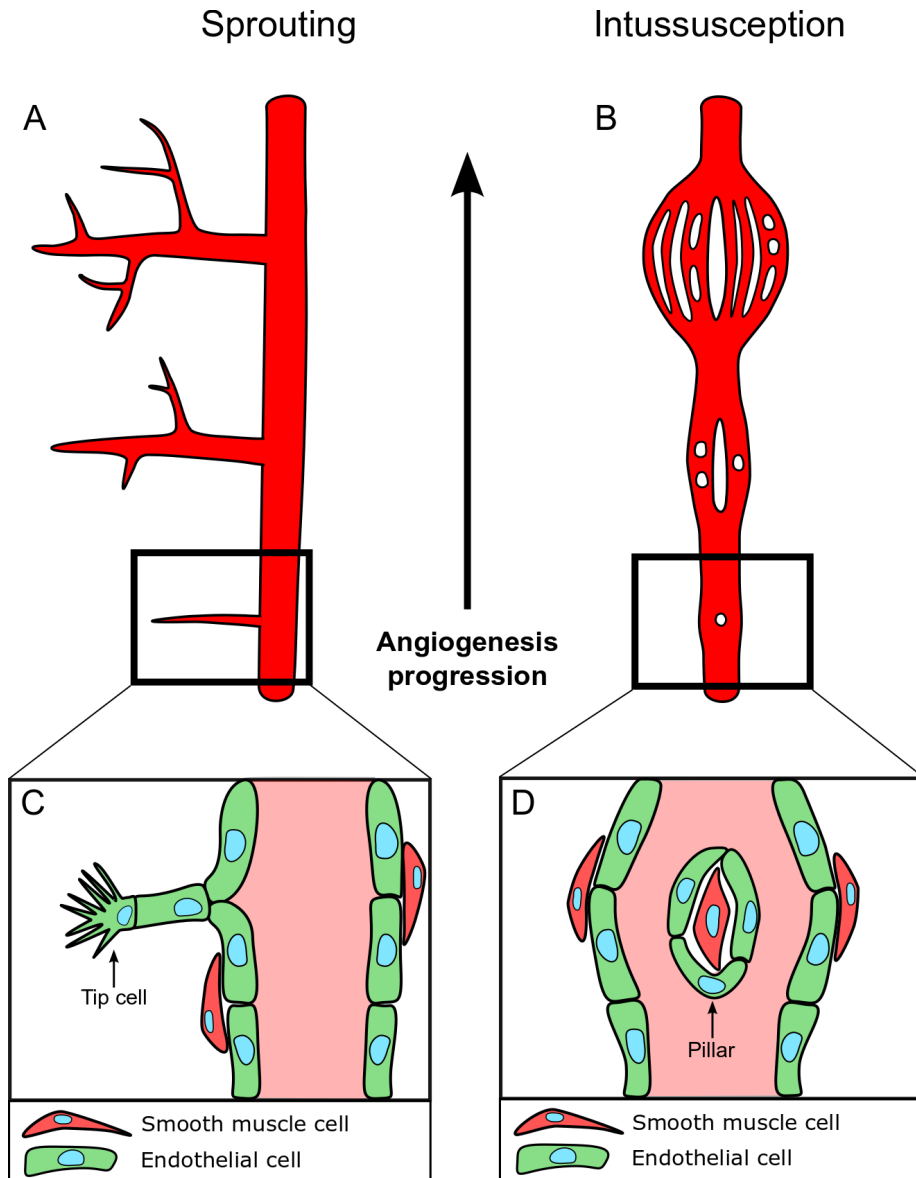
Figure 4 - Schematic image of sprouting angiogenesis and intussusceptive angiogenesis. A. Sprouting angiogenesis is characterized by the outgrowth of a sprout from an existing vessel. B. Intussusceptive angiogenesis forms tissue pillars through the vessel, splitting it into two. C. The sprout is led by a top cell which directs the sprout toward a gradient of VEGF, and the cells of the stalk behind it are proliferative. D. The intussusceptive pillar consists of endothelium connecting both ends of the vessel, forming the outside of the pillar. Smooth muscle cells and collagens are inside the pillar.

1.5.1.2 INTUSSUSCEPTIVE ANGIOGENESIS

Intussusceptive angiogenesis was discovered as a separate form of angiogenesis in 1986 by Burri et al. [77, 78]. However, features of intussusceptive angiogenesis were described already in 1895 in fish gills [79], and in 1950 in embryonic lungs [80].

A literature search reveals that the field of intussusceptive angiogenesis research is small, consisting of less than 200 articles in total. In comparison, sprouting angiogenesis (often just referred to as angiogenesis) has articles published in the magnitude of 20,000-100,000 depending on search terms and location of keywords (Table 3).

Table 3 - Literature search for intussusceptive angiogenesis and sprouting angiogenesis on PubMed and Scopus (accessed on 2019-09-18).

Search term	Location	PubMed	Scopus
"splitting AND angiogenesis" OR "intussusceptive AND angiogenesis"	Title/abstract	178	198
"splitting angiogenesis" OR "intussusceptive angiogenesis"	Title	39	41
"sprouting AND angiogenesis"	Title/abstract	2642	2646
"sprouting AND angiogenesis"	Title	150	158
"angiogenesis"	Title/abstract	90,935	97,984
"angiogenesis"	Title	23,825	26,979

The first step in intussusceptive angiogenesis is characterized by the formation of a thin tissue pillar, which extends across the lumen of the vessel (Figure 4). The pillar is thin, 2-4 µm in diameter. A schematic drawing of the different steps in intussusceptive angiogenesis is shown in Figure 1 of Paper III. The first step of intussusception is the formation of a pillar across the vascular lumen. As the pillar matures, collagen bundles are pulled into the core [81]. Pericytes, smooth muscle cells, and fibroblasts cells then migrate into the pillar to aid in maturation until it resembles the vascular surroundings [82]. More pillars usually form downstream of the initial pillar, which finally fuse to separate the vessel longitudinally into two new vessels [83]. During the process

of intussusceptive angiogenesis, basement membranes must be remodeled by enzymes such as MMPs [84]. Without remodeling, the basement membrane is stiff, causing pericytes, smooth muscle cells, and fibroblasts to be locked in place around the vessel.

Traditionally, intussusceptive angiogenesis has been analyzed by corrosion casting along with scanning electron microscopy (SEM) [78]. This method removes all tissues surrounding the vascular lumen, leaving an imprint of the inside of the blood vessel, enabling identification of pillars. Another method of analyzing intussusceptive angiogenesis is transmission electron microscopy (TEM). TEM requires a large time investment and hundreds of tissue sections and is feasible only in tissues with a high density of intussusceptive angiogenesis [85]. A less time-consuming method of identifying intussusceptive pillars is to use immunofluorescence. By using a combination of markers for endothelium, collagens, and pericytes/smooth muscle, it is possible to identify pillars in a normal fluorescence microscope. Identified pillars may be 3D reconstructed by confocal microscopy to verify the structure since pillars can only be verified by 3D imaging [82].

The mechanisms of intussusceptive angiogenesis are not fully elucidated. Due to pillars forming across a functional vascular lumen, the presence of a growth factor gradient is unlikely, since any growth factor transferred into the blood would immediately be whisked away [85]. There are studies implicating that nitric oxide signaling is involved in the switch from sprouting to intussusception [86], and blood flow dynamics are highly indicative of intussusceptive angiogenesis, with the majority of pillars occurring close to vessel bifurcations [83]. Indeed, intussusceptive angiogenesis is induced in skeletal muscle after exercise or electrical stimulation, both of which increase blood flow and nitric oxide signaling [87, 88]. Other factors thought to be involved in intussusceptive angiogenesis are TIE-receptors [89] and basic fibroblast growth factor (bFGF) [90]. Furthermore, inhibition of Notch signaling has been shown to enhance pillar formation [91], mediated through stromal cell-derived factor-1 (SDF-1, also known as CXCL12) [92] signaling, which mobilizes bone marrow-derived cells (BMDCs). The involvement of BMDCs indicates that a functioning immune system may be required for pillar formation.

It has been suggested that during embryonal and postnatal development, sprouting angiogenesis establishes the vascular network into a growing tissue in an invasive manner, after which the intussusceptive expansion and remodeling phase takes place [93, 94]. Sprouting is slower and more energy-consuming, whereas intussusceptive angiogenesis is faster and more efficient, allowing rapid organ growth.

2 AIMS

The overall aim of this thesis is to study the effects of deranged energy metabolism in atherosclerosis and cancer.

Paper I

- To address the hypothesis that energy failure develops in the core of human advanced atherosclerotic plaques.

Paper II

- To investigate if intracellular and extracellular levels of reactive oxygen species (ROS) within the mouse aorta increase before or after diet-induced lesion formation.
- To investigate if intracellular and extracellular ROS correlates to cell composition in atherosclerotic lesions.
- To investigate if intracellular and extracellular ROS levels within established atherosclerotic lesions can be reduced through lipid-lowering by diet or atorvastatin.

Paper III:

- To address the hypothesis that intussusceptive angiogenesis promotes growth of human tumors.
- To establish a model to elucidate the cellular and molecular mechanisms behind intussusceptive angiogenesis with the further aim of identifying potential drug targets.

3 HYPOXIA, ENERGY METABOLISM, AND ATHEROSCLEROSIS

3.1 THE HEALTHY VASCULAR WALL

Blood vessels are more than just pipes in which blood is transported. Vessels are built from a variety of different cell types, all of which require oxygen and nutrients to function. The vascular wall is divided into the intima, media, and adventitia (Figure 5).

The innermost, intimal, layer (or tunica intima) consists of a single layer of endothelial cells and sub-endothelial tissue. The function of the endothelial cells is to keep the blood inside the lumen and prevent it from coming into direct contact with the tissue. If the endothelium is damaged, blood will clot to cover the wound and repair the damage [95]. The medial layer (or tunica media), consists mainly of smooth muscle cells, elastin, and collagen, which provide contractile force to the vessel [96]. The outermost layer is called the adventitial layer (or tunica adventitia) and consists of collagen and other extracellular matrix components which give stability to the vessel, as well as a point to attach the vessel to other structures. The adventitia also contains blood vessels (vasa vasorum) and progenitor cells [97].

The cells of the vascular wall receive oxygen and nutrients from two sources; directly from blood in the vascular lumen, and from the vasa vasorum [98]. The vasa vasorum is a vascular network in the adventitia and, in large arteries, the outer third of the media [99]. The vasa vasorum are functional end arteries, meaning they have low anastomosis (few cross-connections) and are sensitive to occlusion. Even in the healthy vascular wall, the supply of oxygen and glucose is strained with low concentrations in the mid-media, making the vascular wall vulnerable to perturbations [100, 101].
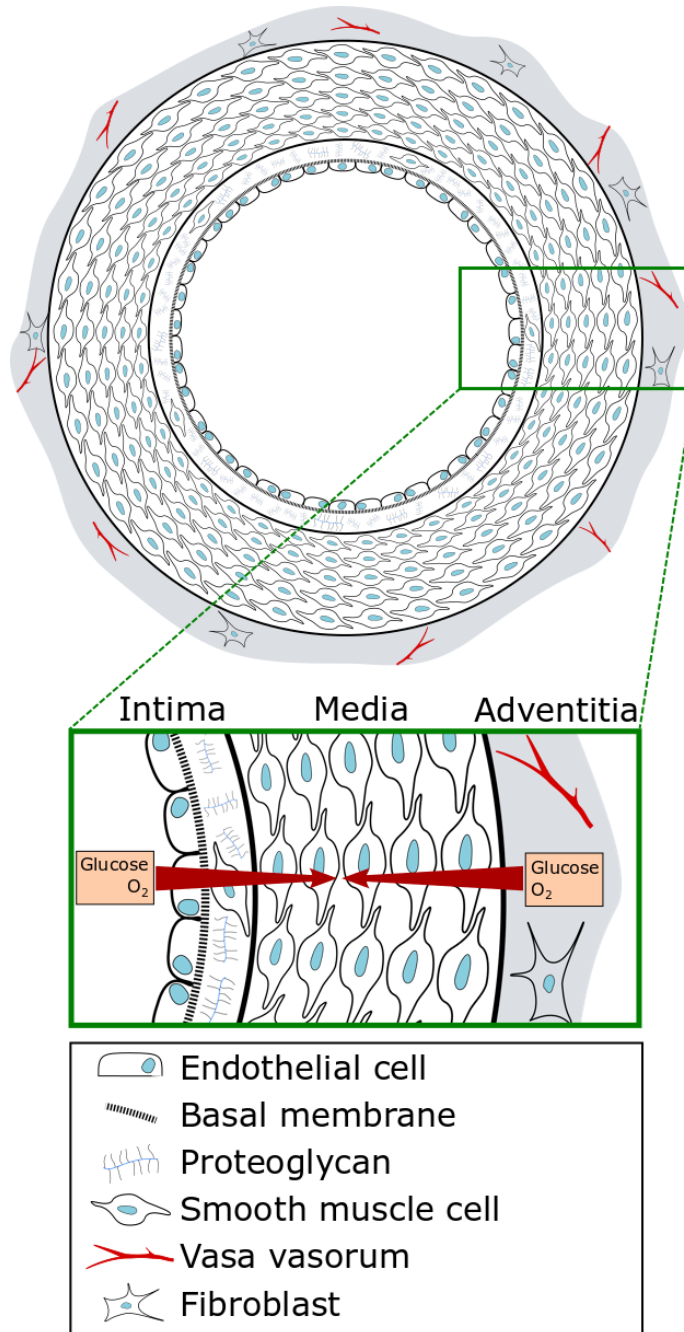
Figure 5 - The healthy vascular wall. Reprinted from Leppänen et al. with permission[102]. Oxygen and nutrients are supplied directly from luminal blood and from vasa vasorum.

3.2   DEVELOPMENT OF ATHEROSCLEROSIS

Atherosclerosis is one of the leading causes of death and disability worldwide [103]. Atherosclerosis is more prevalent in men, who develop it at an earlier age, than women, who women develop atherosclerosis mainly after menopause [104]. Some risk factors which contribute to the development of atherosclerosis are high cholesterol and lipoprotein levels, high blood pressure, smoking, overweight, insulin resistance, and diabetes [105].

Atherosclerosis is characterized by the accumulation of lipids and macrophages in the intimal layer of the vascular wall, causing a narrowing of the vascular lumen by a plaque (Figure 6). The narrowing of the lumen results in impaired blood supply to organs and tissues. If the plaque ruptures in a coronary or carotid artery, the resulting blood clots may cause myocardial infarctions or stroke. Atherosclerosis has a subclinical or "silent" phase with no clinical manifestations, which may last for years or decades [106].

Atherosclerotic plaques are classified according to Stary [107]. Early plaques (stage I and II) contain a few macrophages in the intima and some intracellular lipid accumulation. Intermediate plaques (stage III) have more accumulated extracellular lipids in addition to an increase in macrophages. Advanced plaques (stage IV to VI) contain a pronounced accumulation of macrophages and a necrotic core of extracellular lipids.

3.2.1   INITIATION OF ATHEROSCLEROSIS

One of the initial steps in the development of atherosclerosis is the recruitment of monocytes into the intima. The monocytes differentiate into macrophages, which take up modified lipoproteins containing lipids, and become macrophage foam cells [108]. The theories surrounding the initiation of atherosclerosis have developed over the years, but a common theme is the presence of oxidized LDL.

The response to injury hypothesis postulated in 1977, states that atherosclerosis is initiated by an injury to the vascular endothelium [109]. Examples of injuries are high blood glucose, hyperlipidemia, hypertension, turbulent blood flow, reactive oxygen species, or a combination. The injury results in the recruitment of monocytes to clean up the injury site. The monocytes accumulate lipids, including oxidized LDL, resulting in development of fatty streaks, which is an early event in the development of atherosclerosis. The response to injury hypothesis is however questioned, mainly due to the injury being vaguely specified, and that lipids accumulate before monocyte recruitment in animal models [110].
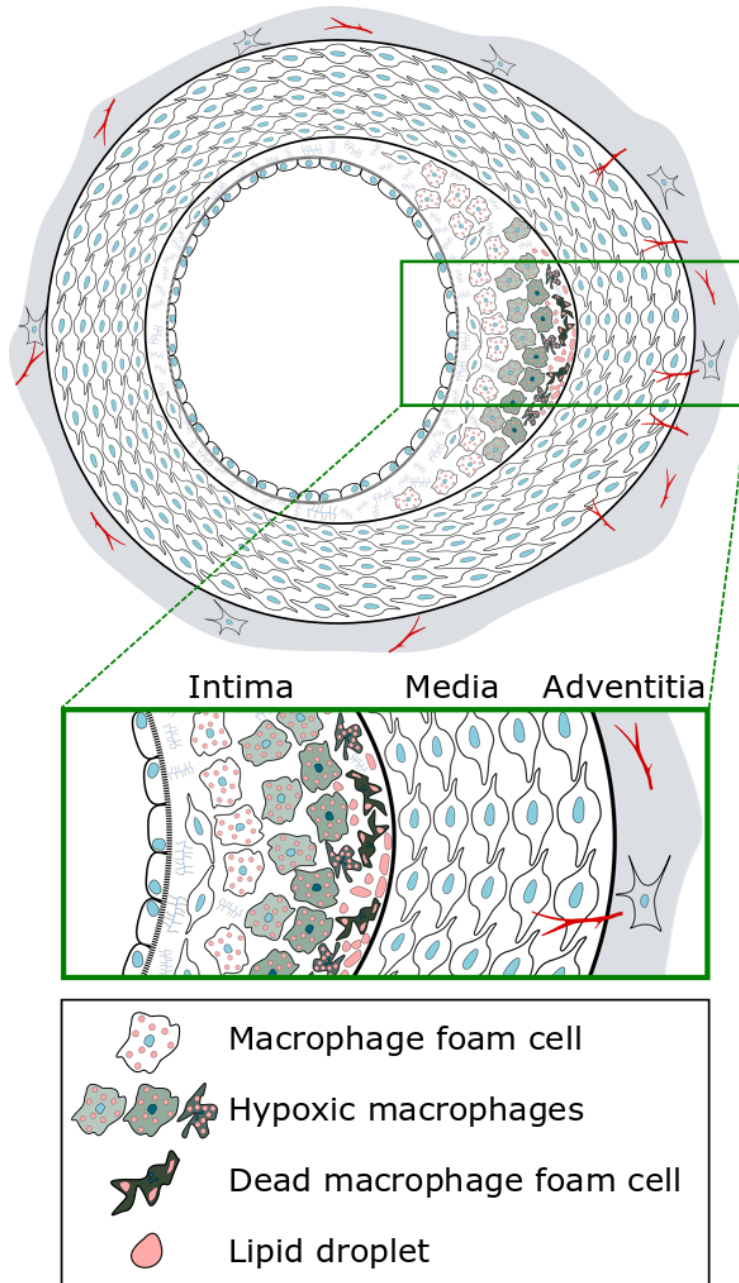
Figure 6 – Advanced atherosclerotic plaque. Modified from Leppänen et al. with permission [102]. Note accumulation of macrophage foam cells and necrotic cells in the core of the plaque.

The oxidative modification hypothesis was postulated in 1989 [111]. It states that oxidized LDL is responsible for recruiting monocytes to the intima. Monocytes phagocytose oxidized LDL and are transformed into macrophage foam cells. A strength of this model is that it does not require endothelial damage, in contrast to the "response to injury" hypothesis.

The response to retention hypothesis postulated in 1995, states that the initiating step of atherosclerosis is the retention of lipoproteins within the vascular wall [112]. Intimal hyperplasia, or diffuse intimal thickening, is thought to expose proteoglycans. Lipoproteins bind to these exposed proteoglycans, thus promoting lipid retention in the vascular wall [113, 114].

Regardless of the exact initiator, a common feature of all these theories of atherosclerosis initiation is an early accumulation of oxidized LDL and macrophages in the intima.

3.2.2 PROGRESSION OF ATHEROSCLEROSIS

The majority of adults have some degree of atherosclerosis [115]. However, most atherosclerotic plaques are early or intermediate plaques which are asymptomatic. Formation of a necrotic core in atherosclerotic lesions is a crucial event because it marks the transition from a stable, clinically silent plaque into an advanced, unstable, plaque which may rupture, causing myocardial infarction or stroke [116, 117]. Therefore, it is of pivotal importance to elucidate the mechanisms promoting the death of macrophage foam cells in the plaque core.

The most abundant cell type in advanced plaques is the macrophage foam cell. Macrophage foam cells contain an abundance of lipids, giving them a foam-like look. Both hypoxia and inflammation trigger macrophages to shift their metabolism toward using glycolysis [118]. Glycolysis is much less energy-efficient and promotes excessive consumption of glucose. Indeed, foam cells have been shown to have excessive consumption of glucose and oxygen [119, 120].

Macrophages in the plaque may die via apoptosis or necrosis. Apoptosis is triggered by internal or external signals for the cell to self-destruct due to an overload of errors in cellular mechanisms or pathological behavior [121]. Apoptosis requires energy in the form of ATP and uses caspases to trigger cell death. A more uncontrolled form of cell death is called necrosis, where the cellular membrane starts leaking, resulting in cellular death. Apoptosis is favored in the presence of high intracellular ATP levels, while necrosis is favored when intracellular ATP levels are low [122]. If apoptotic cells are not properly cleared by other phagocytes, the cellular remains may start leaking,

which results in secondary necrosis [123]. The most common type of cell death in the human plaque core is necrosis [124].

3.2.3   ENERGY DEPLETION HYPOTHESIS

Energy depletion (ATP depletion) in the plaque core may promote macrophage death. The energy depletion hypothesis was initially suggested based on observations of a large number of human plaques [125]. The theory states that when atherosclerosis progresses, the distances to oxygen and nutrient supplies increase. The intimal layer, previously supplied directly by the luminal blood, now have vast distances to oxygen and nutrient supply (Figure 7). In addition to increased diffusion distances, the energy demand of the plaque increases during the progression of atherosclerosis. Accumulation of macrophage foam cells with excessive consumption of both oxygen and glucose [126, 127] may decrease the availability of both oxygen and glucose in the plaque core.
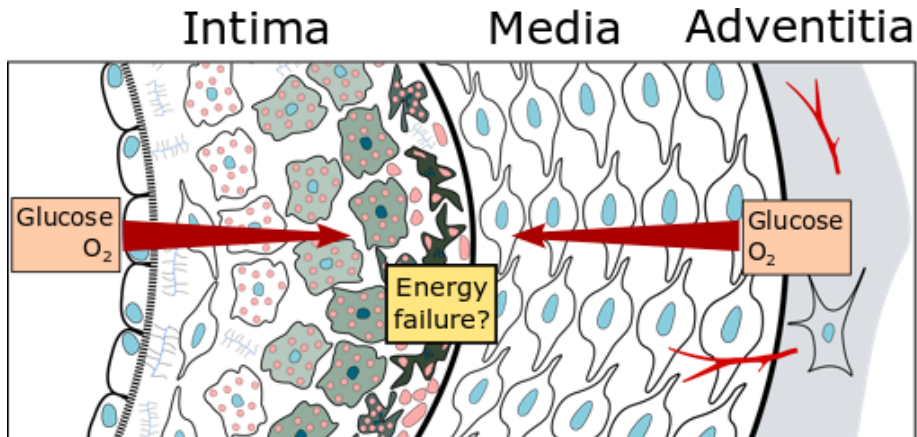
Figure 7 – The energy depletion hypothesis. As diffusion distances increase within the plaque, the cells close to the necrotic core experience energy failure. Reprinted from Leppänen et al. with permission [102].

In support of the energy depletion hypothesis, hypoxia has been demonstrated in the cores of rabbit- [128, 129], mouse- [130] and human plaques [131]. Additionally, glucose and ATP deficiency have been demonstrated in the core of advanced rabbit plaques [126]. A combination of hypoxia and glucose deficiency results in ATP deficiency (137), which may drive necrotic core expansion. However, local concentrations of energy metabolites in different regions of human atherosclerotic plaques are not known.

3.3   PAPER I

In Paper I, the aim was:

- To address the hypothesis that energy failure develops in the core of human advanced atherosclerotic plaques.

In short, advanced plaques were collected from patients and snap-frozen in once piece. Bioluminescence imaging was chosen to measure energy metabolites due to its ability to provide spatial information of energy metabolite distribution [126]. Energy metabolites were measured in intermediate and advanced levels of each patient plaque. Next, levels of energy metabolites were compared assessed in different regions within advanced plaques.

3.3.1   RESULTS

ATP was measured and normalized against the number of cells in the viable intima of each plaque (Figure 8). The ATP/cell in the advanced plaques were only 1/10 of the intermediate plaques, indicating that they are severely ATP-deficient (Figure 9C). The ATP deficiency is supported by a decreased level of extracellular glucose (Figure 9D) and intracellular glycogen (Figure 9E), indicating a deficiency in both energy supply and storage. As a consequence of hypoxia and high glycolysis, lactate levels are increased in the advanced plaques (Figure 9F).
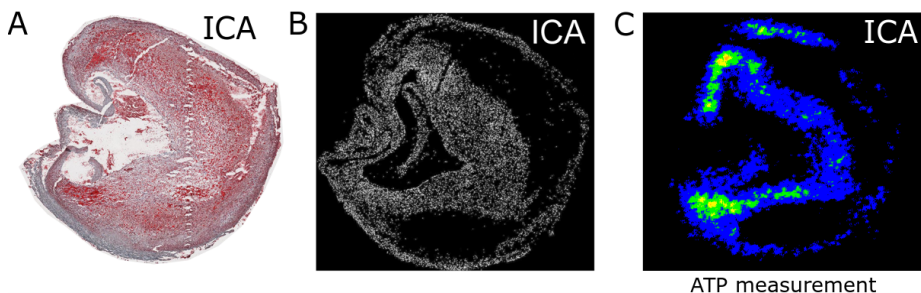
Figure 8 - Method overview. A. Oil red O section of an advanced plaque from the internal carotid artery (ICA). B. cellularity was assessed by fluorescence. C. ATP measurement by bioluminescence (unpublished image). Images assembled from Paper I, Ekstrand et al. [132].
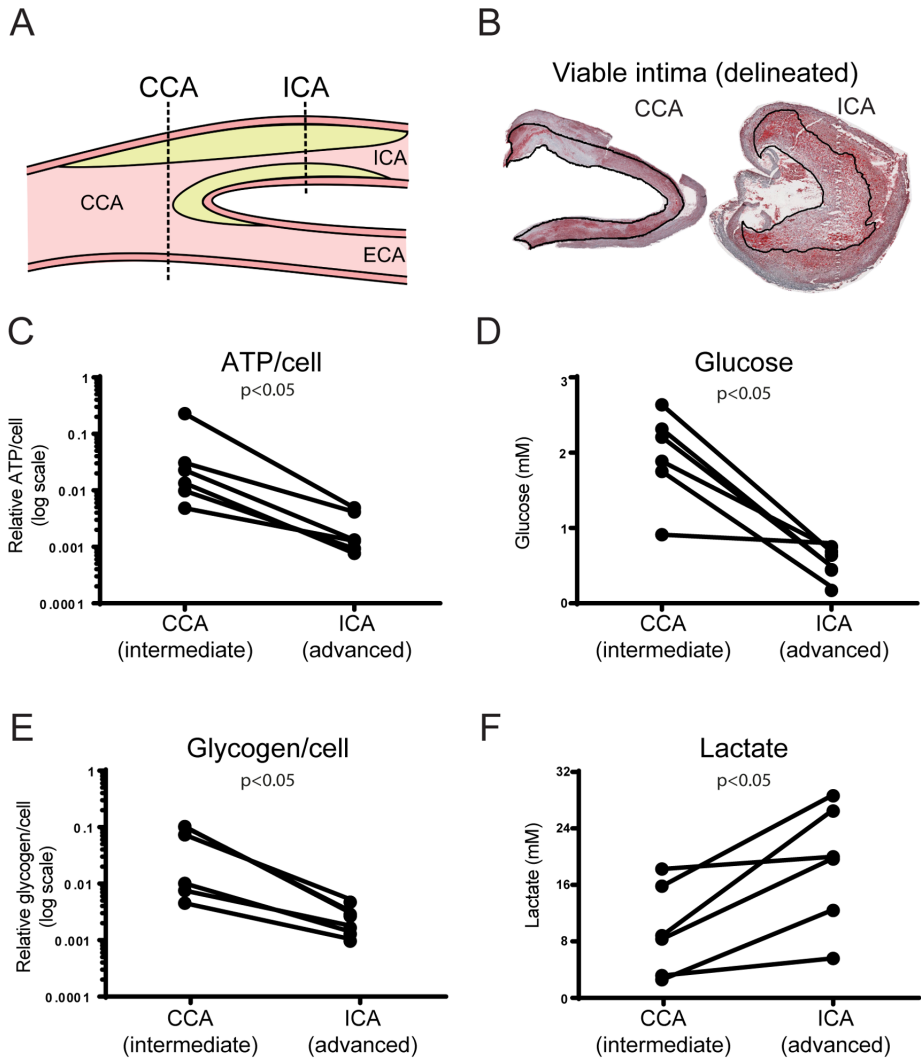
Figure 9 – Figure 2 of Paper I, Ekstrand et al. [132]. Depletion of ATP, glucose, and glycogen in advanced human plaques. A. Plaques were analyzed from the CCA (intermediate) and ICA (advanced). B. Measurements were made in the viable intima of each plaque. ATP/cell (C) glucose (D) and glycogen concentrations (E) were lower in advanced segments than in intermediate segments of the same plaque. Note logarithmic scale for ATP and glycogen. F. Lactate concentrations were higher in advanced segments of the plaque. n = 6, Wilcoxon Signed-Rank Test.

The viable intima, luminal zone, and perinecrotic zones of advanced plaques were marked (Figure 10). ATP/cell and extracellular glucose levels were significantly lower in the perinecrotic zone compared to the luminal zone (Figure 11).
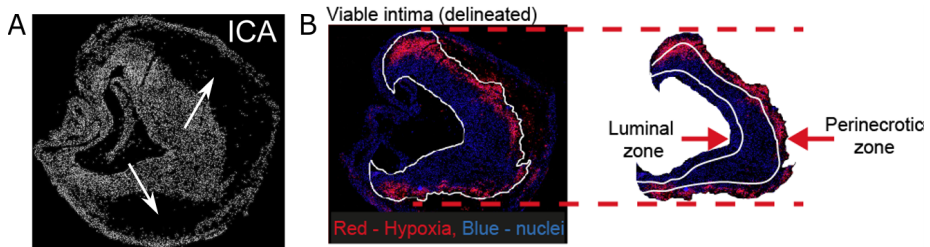
Figure 10 – Necrotic zones were identified (A, white arrows), after which viable intima was delineated (B). In the viable intima, the luminal zone and perinecrotic zones were marked. Images assembled from Paper I, Ekstrand et al. [132].

Figure 11 – Excerpt from Figure 3 of Paper I, Ekstrand et al. [132] Severe depletion of ATP and glucose in perinecrotic zone of advanced plaques. Lower concentrations of ATP (B) and glucose (C) were measured in the perinecrotic zone compared to the luminal zone (p<0.05). n = 6, paired t-test.

3.3.2 DISCUSSION

In Paper I, we demonstrate that energy metabolites (ATP, glucose, glycogen) are depleted in advanced human atherosclerotic plaques. Within advanced plaques, energy metabolites (ATP, glucose) are most depleted close to the necrotic core. This data supports the hypothesis that ATP depletion develops in the core of advanced human atherosclerotic plaques, and may contribute to necrotic core expansion.

We demonstrate reduced availability of glucose and accumulation of lactate in advanced plaques compared to intermediate plaques. In agreement with our results, Tomas et al. [133] performed metabolic profiling of plaque homogenates and demonstrated decreased glucose availability and lactate accumulation in vulnerable plaques. However, ATP levels were not found to be different between vulnerable and more stable plaques in their study. ATP concentrations varied greatly between plaques and spanned a tenfold range. We found a similar wide range of ATP concentrations between plaques in our study (Figure 9C; note logarithmic scale). However, we did find significant differences in ATP levels when intermediate and advanced parts of each patient were compared (Figure 9C). Also, and more importantly, ATP levels were significantly lo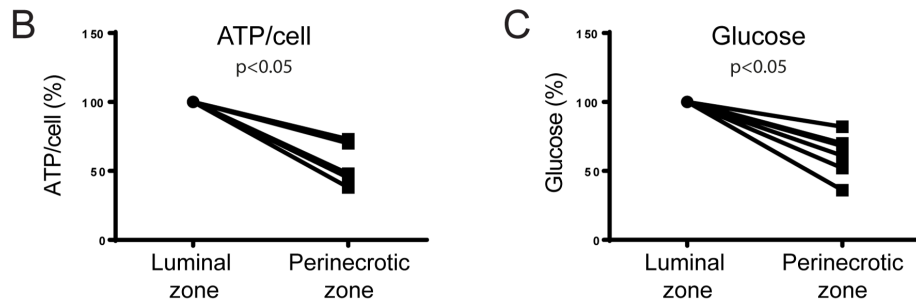wer in the perinecrotic zone compared to the luminal zone within advanced plaques (Figure 11B). The wide range of ATP levels between different patients probably reflects different degrees of ATP hydrolysis depending on the time required for dissection during surgery.

ATP is the fuel driving almost all energy-requiring cellular processes. In atherosclerosis, it is particularly interesting that key events in lipid clearing are directly dependent on ATP, such as reverse cholesterol transport, cellular mobility, and efferocytosis (clearing of dead cells). A reduced reverse cholesterol transport results in an increased cholesterol load on the cells, paradoxically putting them under increased ATP demand where cholesterol is hydrolyzed and re-esterified, consuming 1 ATP per 2 cholesterol every 24 hours [134]. Likewise, a decrease in cellular movement and efferocytosis will decrease the ability of the plaque to be cleared of lipids and cellular debris by outmigration of macrophages. ATP-deficient cells may become necrotic, or promote necrosis over apoptosis [122], thus facilitating necrotic core expansion.

Interestingly, patients with obstructive sleep apnea (OSA) have periods of hypoxia and reoxygenation while sleeping, which causes intermittent hypoxia (IH). Patients with IH are shown to have a higher prevalence of atherosclerosis [135]. Giving rabbits and mice with atherosclerosis hyperbaric treatment (pressure chamber with high oxygen levels) reverses atherosclerosis by

decreasing lipid oxidation, decreasing inflammation, increasing antioxidant levels [136, 137], as well as increasing efferocytosis [138]. Indeed, giving patients with diabetic feet hyperbaric treatment improved their atherosclerosis [139]. These data suggest that hypoxia promotes atherosclerosis progression and that improving plaque oxygenation may reverse atherosclerosis, possibly by restoring ATP levels and energy balance within the plaque. Increased ATP levels would improve the viability of plaque cells, enabling them to perform tasks such as reverse cholesterol transport, efferocytosis, and outmigration, allowing the plaque to heal.

Finally, an increase in lactate levels means the pH of the advanced plaque decreases. A lower pH increases the likelihood of LDL aggregation, fusion and lipid droplet formation [140], which increases its affinity for binding to proteoglycans of the arterial intima [141], which in turn is a driver for atherosclerosis. Acidic pH increases the oxidative modification of LDL and downregulates the expression of ABCA1, which is needed for reverse cholesterol transport [142].

3.3.3 CORRECTIONS

As seen in Figure 1 in Paper I, some statistics were incorrectly based on a Student's T-test, which assumes parametric unpaired data, instead of Wilcoxon signed-rank test, which assumes nonparametric paired data. Corrections for Figure 1 are provided here; Figure 1C macrophages ($p=0.0313$), and smooth muscle cells ($p=0.2188$), Figure 1D cellularity ($p=0.4375$) and necrotic area ($p=0.0313$). None of these corrections change the level of significance, nor the conclusions of the Paper.

3.4 REACTIVE OXYGEN SPECIES IN ATHEROSCLEROSIS

ROS are reactive compounds that are formed during metabolism or by specific enzymes. ROS are part of cellular signaling [44], but an excess may have pathological effects. There are several types of ROS present inside and outside cells, and they are neutralized through different protective mechanisms to limit their potential deleterious effects [44]. Some of the most important enzymes in protecting against ROS are SOD [56], catalase [57], and glutathione peroxidases (GPx) [59, 60].

High ROS levels are present in atherosclerosis and are part of the inflammatory response, oxidative modification of LDL, and proliferation signaling [143]. Depending on the location of excessive ROS, it can have different deleterious effects. Intracellular ROS may damage DNA, proteins, lipids, and carbohydrates [53]. Extracellular ROS oxidizes lipoproteins and activates matrix metalloproteinases [144]. Oxidation of LDL results in an increase of pro-atherogenic properties. Once macrophages detect oxidized LDL, they trigger chronic inflammation, which in turn increases recruitment of immune cells [145]. Extracellular ROS also increases adhesion of leukocytes to endothelium, meaning that immune cells are recruited from the blood to sites where ROS is present [63].

There is no non-invasive technique that allows arterial ROS to be determined *in vivo*. Fixed or frozen material does not reflect the *in vivo* situation since ROS has a half-life of milliseconds. Additionally, current methods to assess arterial ROS do not discriminate between intracellular- and extracellular ROS. We introduce a technique that allows real-time visualization and quantification of ROS in intact aortas incubated under *in vivo* like conditions. Our technique utilizes the ROS-sensitive luminescent probes luminol and isoluminol.

3.5 PAPER II

In Paper II, the aims were:

- To investigate if intracellular and extracellular levels of reactive oxygen species (ROS) within the mouse aorta increase before or after diet-induced lesion formation.
- To investigate if intracellular and extracellular ROS correlates to cell composition in atherosclerotic lesions.
- To investigate if intracellular and extracellular ROS levels within established atherosclerotic lesions can be reduced through lipid-lowering by diet or atorvastatin.

In short, we established a bioluminescence imaging method with ROS probes for intracellular or extracellular ROS measurement in living mouse aortas *ex vivo*. Intracellular and extracellular ROS was correlated against macrophage and smooth muscle cell content. ROS levels were measured before, during, and after atherosclerotic lesion development to assess at which time point ROS levels increase. Finally, atherosclerotic mice were treated for 5 days with high dose atorvastatin or lipid-lowering by diet, after which intracellular and extracellular ROS was measured.

3.5.1 RESULTS

We established a method for assessing intracellular and extracellular ROS separately in living female Apoe$^{-/-}$ mouse aortas (Figure 12). The presence of smooth muscle and macrophages was measured with immunostaining. Intracellular ROS was shown to correlate with macrophages, and extracellular ROS correlates with smooth muscle (data not shown).
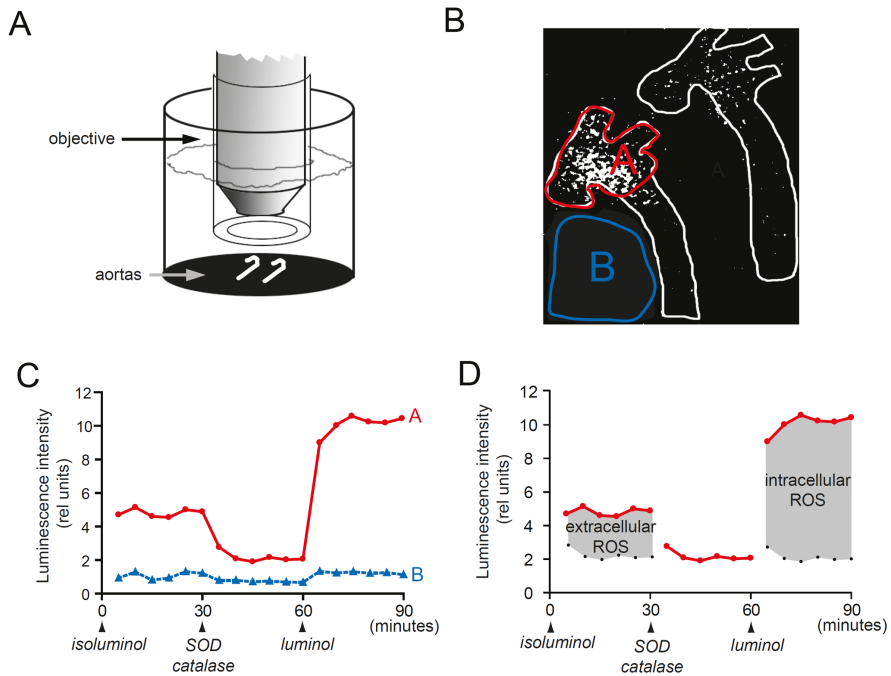
Figure 12 - Figure 1 from Paper II, Ekstrand et al. [146]. A. Living aortas were placed in an organ bath below a photon-counting camera. B. Photon counting images of two aortas. The left aorta has extensive atherosclerosis, while the right one has limited atherosclerosis. C. Luminescence (y-axis) over time (x-axis) in the aortic arch (red line) and background (blue line). Isoluminol is not cell-permeable, while luminol is cell-permeable. D. Addition of SOD and catalase marks quenching of extracellular ROS. Difference between quenched signal and isoluminol signal marks extracellular ROS. Addition of luminol to the quenched extracellular signal marks intracellular signal.

Measuring ROS-levels in the aorta during different times of atherosclerosis development revealed that after 7 weeks of western diet, mice developed visible atherosclerotic lesions that were high in both intracellular and extracellular ROS (Figure 13). After 3 weeks of western diet, before the development of visible atherosclerotic lesions, intracellular ROS was increased (red box), while extracellular ROS was still low (blue box).
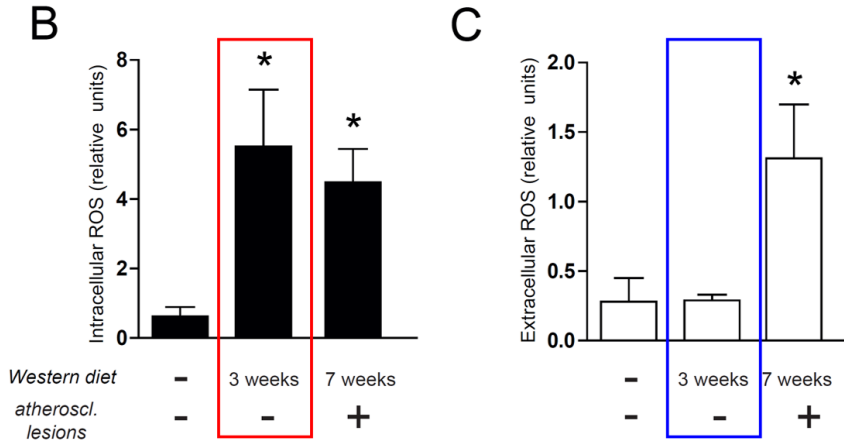
Figure 13 - Excerpt from Figure 2 of Paper II, Ekstrand et al. [146]. After 3 weeks of western diet, intracellular ROS increased despite no visible lesions being present (red box), while extracellular ROS was still low (blue box). n = 6–7 in each group. *p>0.05 vs chow diet, One-way ANOVA with Dunnett's multiple comparison test.

Treating atherosclerotic mice with high-dose atorvastatin for 5 days decreased both intracellular and extracellular ROS levels (Figure 14). This effect does not correlate with either plasma cholesterol nor plasma triglyceride levels. Lipid-lowering by diet for 5 days had no effect on ROS levels within the aortic arch (Figure 15).
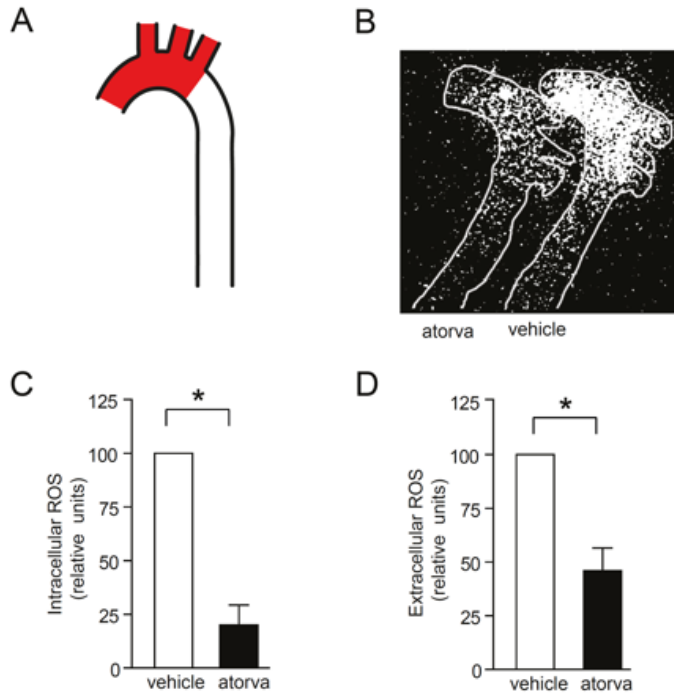
Figure 14 – Excerpt from Figure 6 of Paper II, Ekstrand et al. [146] **Atorvastatin reduces intracellular and extracellular ROS levels within the atherosclerotic aortic arch.** n = 6 in each group. **p<0.01 vs vehicle, *p<0.05 vs vehicle. One sample t-test (C and D).
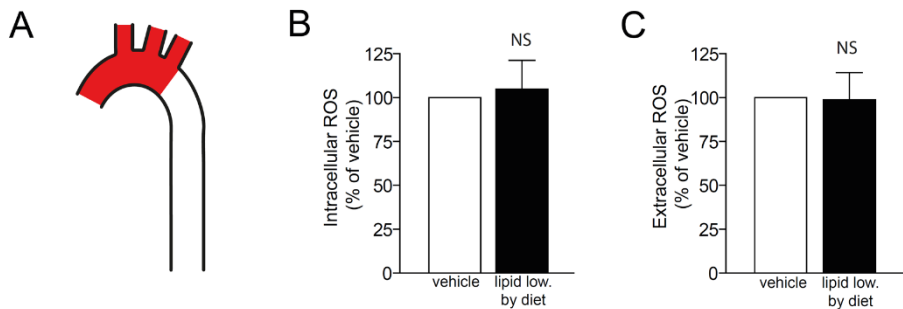
Figure 15 – Figure 4 of Paper II, Ekstrand et al. [146]. **Lipid lowering by diet does not affect ROS levels within the atherosclerotic aortic arch.** (A) Intracellular and extracellular ROS were assessed in the atherosclerotic arch (red). (B) Lipid lowering by diet did not affect intracellular ROS. (C) Lipid lowering by diet did not affect extracellular ROS. NS — non-significant. One sample t-test. n = 6 in each group.

3.5.2 DISCUSSION

In Paper II, we established a method of measuring intracellular and extracellular ROS individually in living mouse aortas *ex vivo*. We demonstrated that intracellular ROS correlates with smooth muscle cells, and extracellular ROS correlates with macrophages. Further, we provided evidence of an increase in intracellular ROS before the development of visible lesions. Finally, short-term atorvastatin treatment decreased both intracellular and extracellular ROS, while lipid-lowering by diet did not.

The increase of intracellular ROS before the development of atherosclerosis in the descending aorta of mice is interesting. This finding is in line with observations that mitochondrial DNA damage occurs before the development of lesions in $Apoe^{-/-}$ mice [147]. Additionally, intracellular ROS in smooth muscle has been connected to the development of atherosclerosis [148]. However, our results connect intracellular ROS to macrophages, and not smooth muscle. An increase of intracellular ROS in macrophages before lesion development could be due to intracellular oxidation of LDL by lipoxygenases such as 15-lipoxygenase-2 (15-LOX-2) [149]. 15-LOX-2 is upregulated in macrophage-rich areas of carotid plaques and is connected to an increased LDL oxidation in macrophages [150]. Further, 15-LOX-2 has been reported to increase expression of ROS via Nox-enzymes [151, 152], possibly making it a part of atherosclerosis development.

The reduction of both intracellular and extracellular ROS by atorvastatin was independent of lipid-lowering, plaque composition, or inflammation. The high dose of atorvastatin and short treatment ensures that overall plaque composition is not changed, and the inflammatory signature of the plaque remained stable (Figure 5 of Paper II). These results are in accordance with previous results by Wassmann et al. [153], where they demonstrate downregulation of Nox1 and upregulation of catalase expression in response to atorvastatin treatment, decreasing the overall ROS presence in atherosclerotic rat aorta.

Our results that lipid-lowering by diet does not change ROS is in contrast to previous research by Aikawa et al. [154], where a lipid-lowering diet decreased ROS in rabbit atherosclerotic aortas. The main difference between our studies is the treatment duration. Aikawa et al. gave a lipid-lowering diet for 16 months, whereas we gave it for 5 days. High lipid levels and LDL binding to the intima are crucial processes in the development of atherosclerosis. However, in the advanced plaque, there already exists a large pool of oxidized LDL, and a decreased influx of non-oxidized LDL is most likely not sufficient to change ROS signaling in 5 days.

4 ANGIOGENESIS IN CANCER

In 1971, Judah Folkman proposed the hypothesis that cancer growth could be stopped by inhibiting blood vessel growth [155]. Despite a massive amount of research into angiogenesis inhibition, current angiogenesis inhibitors have only limited effect in most cancers [156, 157]. However, antiangiogenic drugs work well in many mouse models [158].

There are two forms of angiogenesis, sprouting and intussusceptive angiogenesis. Sprouting angiogenesis is widely studied, whereas intussusceptive angiogenesis is largely enigmatic. Current antiangiogenic drugs target the sprouting pathway, with VEGF and its receptors being the most common targets [159, 160]. It is possible that intussusceptive angiogenesis also promotes the growth of human tumors. However, the role of intussusceptive angiogenesis in human cancer is understudied.

4.1 PAPER III

In Paper III the aims were:

- To address the hypothesis that intussusceptive angiogenesis promotes growth of human tumors.
- To establish a model to elucidate the cellular and molecular mechanisms behind intussusceptive angiogenesis with the further aim of identifying potential drug targets.

In short, biopsies of human malignant melanoma metastases and mouse melanomas were systematically analyzed for the presence of intravascular pillars using epifluorescence and confocal microscopy. In addition, we used a 3D co-culture cell system to study mechanisms of pillar formation with the further purpose of identifying potential inhibitors of intussusceptive angiogenesis.

4.1.1 RESULTS

Strikingly, we found evidence of intussusceptive angiogenesis in all human melanoma metastases in our study (Figure 2 and Figure 3 of Paper III). Our finding indicates that angiogenesis in human tumors is, partly, dependent on a previously virtually ignored mechanism.

Next, we studied the presence of intussusceptive angiogenesis in two mouse models of malignant melanoma, namely a PDX (Patient-Derived Xenograft) [161] model, and a BPT ($Braf^{CA/+}Pten^{f/f}Tyrosinase$-$Cre$) model [162]. No signs of intussusceptive pillars were detected in either mouse model (Figure 4 of Paper III).

Finally, we established a 3D co-culture model which spontaneously develops pillars (figure 5 of Paper III). Using MMP inhibitors, pillar formation is stopped, and smooth muscle cell movement inside pillars was impaired (Figure 6 of Paper III).

4.1.2 DISCUSSION

We demonstrate signs of intussusceptive angiogenesis in human malignant melanoma metastases. Previously, evidence of intussusceptive angiogenesis in human cancer was weak, consisting only of intraluminal structures or vascular invaginations shown by brightfield microscopy [163-165]. Having identified and 3D-reconstructed intussusceptive pillars, they contain the same elements as those that form during development. In this regard, our study provides the first verification of intussusceptive angiogenesis in human cancer.

While we can show the presence of intussusceptive angiogenesis, we cannot currently quantify the extent or importance of it. However, the bare fact of its existence as a parallel mechanism to sprouting angiogenesis in cancer is reason enough to treat it as a major player in resistance against current antiangiogenic therapy.

The fact that no pillars were found in two mouse melanoma models needs to be addressed. There are fundamental differences between mouse models of cancer and human cancer since VEGF inhibitors work well in mice but have marginal effects in humans [158]. One of the mouse models tested is a patient-derived xenograft (PDX) [161] model, which lacks an immune system. While the lack of an immune system is a prerequisite for the model as such to work, it may also interfere with the potential need of recruiting bone marrow-derived cells (BMDCs) for intussusceptive angiogenesis to take place [92]. In the genetic $Braf^{CA/+}Pten^{f/f}Tyrosinase$-$Cre$ (BPT) model [162], tumor formation is genetically triggered, after which tumors rapidly grow and metastasize. This rapid growth may result in tumors not vascularized enough to trigger the intussusceptive phase of angiogenesis. Having access to a mouse model of cancer containing intussusceptive angiogenesis should be a priority for future research on angiogenesis inhibitors.

3D cell culture models are being used more commonly within research [166]. To study the mechanisms of pillar development, we 3D co-cultured endothelium and smooth muscle together [167], enabling them to express basic cellular mechanisms which would be impossible in either monoculture or 2D-cell culture. When cultured together, the cells create tubes (reminiscent of vasculogenesis), tip cells (reminiscent of sprouting angiogenesis), as well as pillars (reminiscent of intussusceptive angiogenesis). We theorize that the endothelial monolayer on top of the smooth muscle mimics the inside of a blood vessel, enabling the cellular program for pillar formation. Since the pillars in our cell model are structurally similar to the intussusceptive pillar, they may share basic cellular mechanisms for their formation, making the

system suitable for studying basic mechanisms of intussusceptive angiogenesis.

In summary, in this paper, we describe the first verified proof for intussusceptive angiogenesis in human malignant melanoma. The presence of intussusceptive angiogenesis in human cancer indicates the need for new therapeutics which target not only sprouting angiogenesis but also intussusceptive angiogenesis in combination. Developing a mouse model reflecting human cancer angiogenesis is essential to studying the antiangiogenic treatment of the future, and suggestions for mechanisms to target can be found in our cell model of intussusceptive angiogenesis.

5 METHODOLOGICAL CONSIDERATIONS

In this section, I will briefly discuss the selection of methods used in the different Papers.

5.1 BIOLUMINESCENCE IMAGING

Bioluminescence is the emission of light from living organisms [168]. Bioluminescence imaging involves using bioluminescent enzymes to visualize substrates within living tissue or animals (Figure 16).
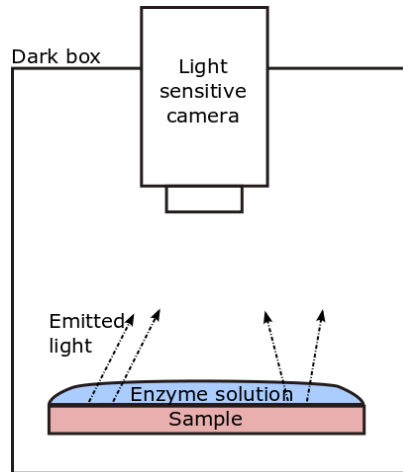
Figure 16 - Schematic image of bioluminescence imaging.

5.1.1 BIOLUMINESCENCE IMAGING OF ENERGY METABOLITES

Bioluminescence imaging of energy metabolites enables the quantification of energy metabolites in frozen tissue sections with a resolution of 50-100 µm [102]. It is, in essence, an *in vitro* method but analysis of snap-frozen tissue reflects the *in vivo* situation. The advantage of the technique, compared to analysis of tissue homogenates, is the high spatial resolution. Bioluminescence imaging of energy metabolites utilizes enzymes such as luciferase from fireflies or bacteria to produce light [102, 169].

In Paper I, we used bioluminescence imaging of energy metabolites on heat-inactivated sections from human atherosclerotic plaques. The measurement of energy metabolites uses a mix of enzymes and substrates to accommodate the breakdown of the energy metabolite of interest, resulting in the release of

photons (Figure 17). A common combination of enzymes containing bacterial luciferase, G6PDH, and NAD(P)H-FMN oxidoreductase, which is used in several pathways, is marked in light blue.
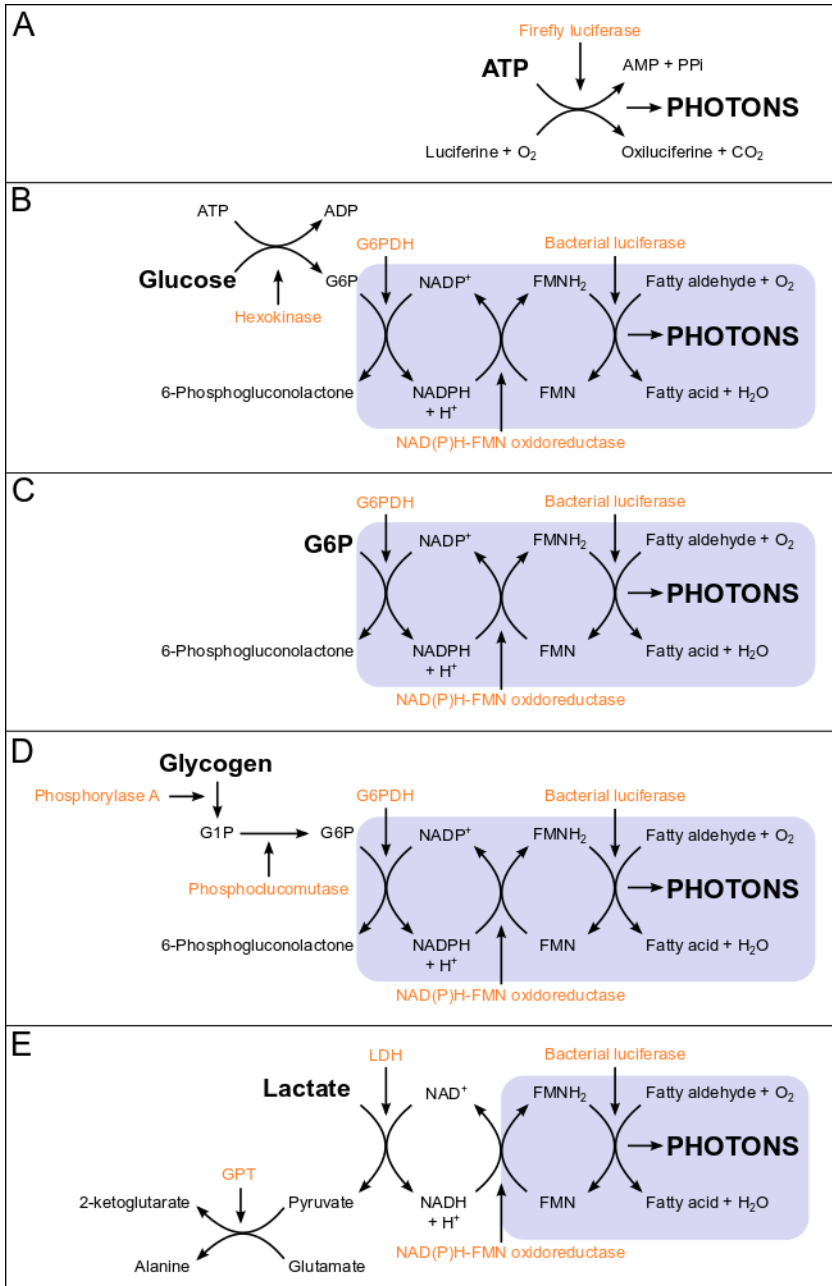

Figure 17 - Enzymatic pathways used for measuring energy metabolites in bioluminescence imaging of energy metabolites. Enzymes are colored orange.

5.1.2 BIOLUMINESCENCE IMAGING OF REACTIVE OXYGEN SPECIES

In Paper II, the ROS probes luminol and isoluminol were used to measure intracellular and extracellular ROS, respectively, in living mouse aorta *ex vivo*. The probes emit photons when they are oxidized by ROS, which can then be registered by the photon camera.

Luminol and isoluminol are probes for ROS, reacting with superoxide [170], hydrogen peroxide [171], hypochlorous acid [172], peroxynitrite [173], and peroxyl radicals [174].

Luminol pass cell membranes (luminol), while isoluminol does not, which enabled individual measurement of intracellular- and extracellular ROS in the same sample. Correction for background signal was made by adding SOD and catalase to scavenge extracellular ROS, enabling quantification of intracellular and extracellular signals separately. Ideally, the same procedure should be used for intracellular ROS signal; however, delivering SOD and catalase intracellularly through liposomes, in our experience, results in clouding of the medium, distorting ROS measurements.

5.2 ANIMAL MODELS

In Paper II, we used female Apoe$^{-/-}$ mice to study ROS during the development of atherosclerosis. Apoe$^{-/-}$ mice are deficient in apolipoprotein E (ApoE), used in lipoprotein transport, and the ApoE deficiency results in hypercholesterolemia [175].

Mouse dietary models of atherosclerosis do not develop advanced lesions [176, 177]. Therefore, genetic models are used to study atherosclerosis development [178]. Since mouse plaques are smaller than human plaques, hypoxia is less prevalent due to shorter diffusion distances, and neovascularization of the intima is uncommon [176] while it is common in human plaques [179], making the study of energy metabolism challenging. Additionally, mouse plaques are rarely unstable [180].

In Paper III, we studied angiogenesis in two mouse models of malignant melanoma to assess the presence of intussusceptive angiogenesis.

The first model studied was a PDX model [161]. In the PDX model, mice without a functioning immune system are injected with human tumor cells subcutaneously, which subsequently grew as a single tumor. The major strength of this model is that the cancer cells are human, and the drawback is

the lack of an immune system. The lack of an immune system may interfere with intussusceptive angiogenesis due to the lack of recruitment of BMDCs, which may be required for intussusceptive angiogenesis [92].

The second model studied used a $Braf^{CA/+}Pten^{f/f}Tyrosinase-Cre$ (BPT) model [162]. In the BPT model, tumor formation is initiated by the topical application of 4-hydroxytamoxifen, which genetically activates BRAF^{600VE} expression and inactivates PTEN expression. This makes melanocytes acquire tumor characteristics, inducing a rapid growth and formation of metastases. The benefit of this model is that tumor cells behave like human tumors, forming metastases. The drawback is that the model is based on a few key mutations, while normal tumors have thousands of mutations [181]. Additionally, the rapid growth of the tumors is possibly causing them not to be vascularized enough to enter the intussusceptive phase of vessel expansion.

5.3   3D CELL CULTURE MODELS

Cell culture models are useful for studying basic cell behavior and dynamics in an artificial environment *in vitro* (in the glass). *In vivo* (within the living), cells grow in a three-dimensional manner and interact with a variety of other cells, both sending and receiving signals that affect cell behavior [182].

The most basic cell culture systems are 2D. All cells are polarized toward the plastic surface of the dish in which they grow, making cell-cell interactions very limited. 2D cell cultures also prevent the cells from forming the structures they do *in vivo*, such as spheres [183] and tubes [184], which is possible in 3D cell cultures. The main benefit of 3D cell cultures is that cells behave more *in vivo*-like compared to 2D cell cultures.

Traditionally, cells have been cultured in a monoculture, consisting of only one cell type. Most organs in the body consist of two or more cell types which interact to achieve both form and function [185]. Culturing two or more cell types together is called co-culture, and doing so brings the system closer to how the cells would behave *in vivo* [186]. By combining 3D cell culture and co-culture, we get a 3D co-culture model where two cell types are free to interact in any spatial direction.

5.4   LIVE-CELL IMAGING

Live-cell imaging, or time-lapse microscopy, is a system where images of cells are taken at certain intervals to track movement and other events during a longer period [187]. Live-cell imaging is a useful tool to understand cellular interactions and movement since a still image provides no information on the cell migration pattern, or how it is interacting with other cells. A series of still images can be made into a movie, which enables visual observation of cellular behavior.

Live-cell imaging requires cells to be placed under a microscope during an extended time. Microscopes are normally not contained within a fully functional cell culture incubator, meaning that cells in live-cell imaging are in an environment different from their normal culture conditions. A live-cell imaging microscope may include a makeshift incubator to support temperature control, and in some cases, even a controlled atmosphere. In our 3D co-culture experiments, cells are contained within a temperature-controlled incubator with normal atmospheric composition. The pH-regulation normally supported by the high carbon dioxide levels of cell culture incubators was instead supported by the addition of HEPES to the cell culture medium.

During live-cell imaging, cells are repeatedly exposed to light of different wavelengths to visualize Celltracker Green and Orange. This light exposure may have some effect on cell viability or behavior of the cells, seeing as most live-cell imaging include the risk of phototoxicity [188]. However, any potential phototoxicity should be similar between the groups used in our experiments.

5.5   IMMUNOFLUORESCENCE

Immunofluorescence is a method where antibodies labeled with fluorochromes directly or indirectly are used to label specific structures in a section of tissue or cells. The labeling can be identified through the use of epifluorescence microscopy (two dimensional) or confocal microscopy (three dimensional), to explore the distribution of these structures of interest in comparison to each other. Immunofluorescence is often coupled with image analysis to quantify the captured signals.

6 CONCLUSIONS AND CLINICAL PERSPECTIVES

This thesis has explored some of the effects that originate from hypoxia, deranged energy metabolism, reactive oxygen species, and angiogenesis.

In Paper I, we present evidence of energy deficiency in advanced human atherosclerotic plaques, and the energy deficiency was most severe close to the necrotic core. This knowledge can be used to target energy-consuming processes or increase energy supply to aid healing of the plaque.

In Paper II, we present evidence of ROS-levels rising before the development of visible atherosclerotic lesions in mice, and that atorvastatin has a ROS-lowering effect which is independent of lipid levels. This knowledge may be used to better understand how atherosclerosis develops and the mechanisms behind the atheroprotective effect of atorvastatin.

In Paper III, we present the first evidence of intussusceptive angiogenesis in human cancer. This knowledge could help explain the limited clinical effectiveness of current angiogenesis inhibitors which target only sprouting angiogenesis. Combining the inhibition of both sprouting and intussusceptive angiogenesis should be explored as a treatment option to prevent tumor growth.

In summary, this thesis provides new insights into the effects of hypoxia and deranged energy metabolism. In combination with future research, these results may help establish new treatments for atherosclerosis and cancer.

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Stefanie Fruhwürth – Quack.

Urszula Chursa – It's good to be a unicorn.

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Oskar Henriksson – … and we need to tax those robots.

John Leander & Boris Pendic – Pharmacist squad.

Jing Yang – Hello there :-)

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APPENDIX

Appendix I – Paper I

Depletion of ATP and glucose in advanced human atherosclerotic plaques

Ekstrand M, Widell E, Hammar A, Akyürek LM, Johansson M, Fagerberg B, Bergström G, Levin MC, Fogelstrand P, Borén J, Levin M.

PLoS One. 2017 Jun 1;12(6):e0178877. PMID: 28570702

Appendix II – Paper II

Imaging of Intracellular and Extracellular ROS Levels in Atherosclerotic Mouse Aortas Ex Vivo: Effects of Lipid Lowering by Diet or Atorvastatin.

Ekstrand M, Gustafsson Trajkovska M, Perman-Sundelin J, Fogelstrand P, Adiels M, Johansson M, Mattsson-Hultén L, Borén J, Levin M.

PLoS One. 2015 Jun 22;10(6):e0130898. PMID: 26098110

Appendix III – Paper III

Intussusceptive angiogenesis in malignant melanoma

Ekstrand M, Pandita A, Bjursten S, Ekelund E, Fogelstrand P, Le Gal K, Nilsson J, Ny L, Johansson I, Bergö M, Akyurek LM, Levin MC, Boren J, Ewald AJ, Mostov K, Levin M.

Manuscript