Metabolic abnormalities shared by macrophages from rheumatoid arthritis and coronary artery disease patients promote inflammation

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Zusammenfassung

dieser Promotionsarbeit mit dem übersetzten Titel:

Der gesteigerte Metabolismus von Makrophagen fördert entzündliche Prozesse bei Patienten mit rheumatoider Arthritis und koronarer Herzerkrankung

Hintergrund und Ziel: Das Risiko für kardiovaskuläre Komplikationen ist bei Patienten mit rheumatoider Arthritis (RA) im Vergleich zur Normalbevölkerung deutlich erhöht und die koronare Herzkrankheit (KHK) ist die häufigste Todesursache dieser Patienten. Bereits in der frühen Krankheitsphase besteht bei RA-Patienten ein erhöhtes Risiko, einen Myokardinfarkt zu entwickeln. Die molekularen Mechanismen, die diesem Zusammenhang zugrunde liegen, konnten bislang nicht vollständig geklärt werden.
Als wichtige Ursache für die Entwicklung von Arteriosklerose und instabilen Gefäßplaques wird zunehmend eine Störung des Immunsystems gesehen und es wird vermutet, dass eine Verbindung zwischen der Inflammation bei RA und der Gefäßentzündung bei KHK besteht. Eine Schlüsselrolle bei der Immunpathogenese von RA und KHK kommt den inflammatorischen Makrophagen zu, welche entscheidend sowohl zur Verletzung der Synovialmembran bei RA als auch zur Bildung von instabilen atherosklerotischen Plaques beitragen.
Die Forschung der letzten Jahre zeigt, dass die immunologische Funktion von Makrophagen stark durch den Metabolismus dieser Zellen beeinflusst wird. Das Ziel dieser Doktorarbeit war, den Metabolismus durch eine Analyse der Glykolyse und der mitochondrialen Atmungskette in Makrophagen von Patienten mit RA oder KHK zu untersuchen und anschließend die Wirkung von den festgestellten Veränderungen des Metabolismus auf die Immunreaktion der Makrophagen zu analysieren.

Methoden: Blutproben für die Experimente wurden von Patienten mit RA (anti-CCP- oder Rheumafaktor-Positivität) oder KHK (mindestens ein Myokardinfarkt in der Vergangenheit) gewonnen. Zusätzlich wurden Proben von einer gesunden Kontrollgruppe entnommen. Aus diesen Blutproben wurden mononukleäre Zellen des peripheren Blutes (PBMC) isoliert, CD14+ Monozyten separiert, zu Makrophagen differenziert und anschließend aktiviert. Die Glykolyse,

mitochondriale Atmungskette und Zytokinausschüttung dieser stimulierten Makrophagen wurden durch quantitative Echtzeit-PCR gemessen. Für die Funktionsanalyse der Atmungskette wurden das mitochondriale Membranpotential und die mitochondrialen Sauerstoffradikale mittels Durchflusszytometrie quantifiziert. Ergänzend wurde intrazelluläres ATP durch einen Lumineszenztest bestimmt. Eine Inhibition von Sauerstoffradikalen wurde durch einen speziellen Radikalfänger (TEMPOL) durchgeführt.

Ergebnisse und Beobachtungen: Im ersten Teil wurden die Glukoseaufnahme und -verwertung sowie die mitochondriale Atmungskette untersucht. Dabei zeigte sich eine signifikante Hochregulation der Gene des *Glukosetransporters Typ 1* (GLUT1) und der Enzyme *Hexokinase* (HK) und *Laktat-Dehydrogenase* (LDH) in Makrophagen von RA- und KHK-Patienten im Vergleich zu der gesunden Kontrollgruppe. Ebenfalls zeigte sich eine erhöhte Genexpression von Proteinen, die für den Elektronentransport und die ATP-Synthese innerhalb der Atmungskette zuständig sind. Das mitochondriale Membranpotential sowie intrazelluläres ATP waren in Makrophagen von RA- und KHK-Patienten im Vergleich zur gesunden Kontrollgruppe signifikant erhöht. Im zweiten Teil wurde gezeigt, dass Makrophagen von RA- und KHK-Patienten vermehrt Sauerstoffradikale sowie erhöhte mRNA-Mengen der inflammatorischen Zytokine IL-6 und IL-1β produzieren. Die Inhibition dieser Sauerstoffradikale führte zu einer Reduktion der Genexpression von IL-6 und IL-1β.

Schlussfolgerungen: Die Ergebnisse zeigen, dass Makrophagen von RA- und KHK-Patienten einen ähnlichen metabolischen und immunologischen Phänotyp aufweisen. Es zeigte sich in den Makrophagen beider Krankheitsbilder sowohl ein erhöhter Glukosestoffwechsel also auch eine erhöhte Aktivität der mitochondrialen Atmungskette. Diese Hochregulation führte zu einer Steigerung der mitochondrialen Sauerstoffradikalbildung, welche für die exzessive Zytokinproduktion dieser Zellen verantwortlich war. Die Erkenntnisse dieser Arbeit zeigen, dass der fehlregulierte Metabolismus in Makrophagen beider Erkrankungen die immunologische Potenz dieser Zellen steuert und eine Erklärung für das erhöhte Risiko kardiovaskulärer Komplikationen bei RA-Patienten sein könnte.

Teilergebnisse der vorliegenden Arbeit wurden veröffentlicht in:

Zeisbrich, M., R. E. Yanes, H. Zhang, R. Watanabe, Y. Li, L. Brosig, J. Hong, B. B. Wallis, J. C. Giacomini, T. L. Assimes, J. J. Goronzy and C. M. Weyand (2018). "Hypermetabolic macrophages in rheumatoid arthritis and coronary artery disease due to glycogen synthase kinase 3b inactivation." Ann Rheum Dis 77(7): 1053-1062.

Abstract

Cardiovascular complications represent the leading cause of premature mortality in patients with rheumatoid arthritis (RA). The risk for myocardial infarction (MI) of RA patients is almost twofold higher compared to the general population. Inflammatory macrophages are key effector cells in the immunopathology of atherosclerotic plaques and rheumatoid joints. They are a major source of inflammatory cytokines and facilitate tissue damage, which leads to joint destruction in RA and atherosclerotic plaque destabilization in coronary artery disease (CAD) patients.

In the conducted experiments monocyte-derived macrophages from RA and CAD patients presented increased expression of genes with pivotal roles in glycolysis and the mitochondrial respiratory chain compared to macrophages from a healthy control group. A functional assessment of the mitochondrial respiratory chain revealed an upregulated mitochondrial membrane potential and increased intracellular ATP production in patient-derived macrophages. Furthermore, these macrophages generated increased levels of mitochondria-derived ROS and IL-1β and IL-6 mRNA. Transcription of these proinflammatory cytokines was dependent on ROS, as ROS-inhibition reduced mRNA of both, IL-1β and IL-6. Throughout the experiments, a hierarchy among the phenotypes of RA and CAD macrophages was observed. CAD macrophages showed a higher metabolic state, greater increase in mitochondrial ROS generation and higher gene expression of cytokines than RA macrophages. In conclusion, monocyte-derived macrophages from RA and CAD patients shared a metabolic phenotype of upregulated glycolysis and mitochondrial activity. High activity of mitochondria led to increased generation of mitochondrial ROS, which upregulated gene expression of proinflammatory cytokines. These findings show that a dysregulated cellular metabolism is shared by macrophages from RA and CAD patients, a finding that might help understanding the increased risk of cardiovascular complications in RA patients.

1 Introduction

1.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease. It leads to inflammation of the synovium (synovitis) that causes cartilage and bone destruction. RA has a prevalence of about 0.5 - 1 % with three-fourths of the patients being female and represents a serious burden for patients and society (Cross et al. 2014, Okada et al. 2019). Smoking and genetic variants, most importantly in the HLA-DR4 gene, are associated with the development, the severity and outcome of RA (Firestein and McInnes 2017, Ospelt et al. 2017).

Inflammation within the synovium leads to recruitment of immune cells from the blood vessel as well as activation of local inflammatory cells. Chemokines, growth factors and especially cytokines such as IL-1β, tumor necrosis factor (TNF) -α and IL-6 have a central role in the immunopathogenesis of RA. Macrophages, neutrophils, lymphocytes, fibroblasts and cells of bone metabolism are activated and contribute to the inflammation and tissue damage. Synovial fibroblasts and osteoclasts are responsible for the damage of cartilage and bone of the affected joints and can lead to irreversible joint destruction (Neumann et al. 2018).

RA patients suffer under symmetrical, painful and disabling joint inflammation years after a dysfunction of the immune system has started (Mankia and Emery 2016, Dekkers et al. 2017). In earlier stages of the disease, mainly smaller joints typically metacarpophalangeal (MCP), proximal interphalangeal (PIP) and metatarsophalangeal (MTP) are affected. The joints are tender, swollen, warm and stiff. The stiffness is worse after inactivity or in the morning. These symptoms often spread to other joints, such as knees, elbows, hips and shoulders, as the disease progresses (Jutley et al. 2017).

About 40 % of the patients suffer under systemic complications, such as cardiovascular disease, osteoporosis, anemia and exudative pericarditis. These extra-articular manifestations contribute to the higher mortality and morbidity in patients with RA (Cojocaru et al. 2010).

1.1.1 Cardiovascular disease – a major complication of rheumatoid arthritis

Despite enormous improvements in the therapy of RA over the previous decades, the life expectancy of RA patients is lower than compared to general population (Romano et al. 2018). The premature mortality in RA patients is mainly attributable to its most severe complication, cardiovascular disease (CVD) (Maradit-Kremers et al. 2005), which accounts for about 50 % of premature deaths in this population (Avina-Zubieta et al. 2008). RA patients have an increased risk for atherosclerosis, the main cause of coronary artery disease (CAD), and their risk for myocardial infarction (MI) is almost twofold higher compared to the general population (Peters et al. 2009). CAD in RA patients presents less typical symptoms, a worse prognosis and is possibly not treated as aggressively as in non-RA patients (Maradit-Kremers et al. 2005, Davis et al. 2008).

A link between RA and the increased risk of CAD among these patients presents the inflammatory component of both diseases (Hansson and Hermansson 2011, Bories and Leitinger 2017). Inflammation plays a central role in instable atherosclerotic plaque development (Taleb 2016), which is one of the most crucial factors of the higher rates and worse outcomes of MIs in RA patients (Aubry et al. 2007). In addition, even an elevated plasma concentration of c-reactive protein (CRP) in healthy men was associated with increased risk for cardiovascular events (Ridker et al. 1997).

Thus, focusing on traditional risk factors for CAD only, such as hypertension, obesity, smoking or diabetes, does not provide a sufficient foundation for CAD risk estimation in RA patients. Additional RA related factors, predominantly the chronic inflammatory state of the disease, need to be considered. HLA–DRB1 variants, intake of glucocorticoids and presence of RA antibodies such as antinuclear antibodies and rheumatoid factors contribute to CAD risk in RA patients (Liang et al. 2009, Jagpal and Navarro-Millan 2018). For better risk assessment of cardiovascular disease for RA patients, EULAR suggests the use of traditional scores such as SCORE and Framingham and to multiple them by a factor of 1.5 for these patients. This advanced CAD risk assessment should be done at least every five years or more frequently when the patient's anti-rheumatic therapy regime is modified (Agca et al. 2017).

In order to lower the risk for CAD in RA patients, a control of traditional CAD risk factors, management of RA disease activity and lifestyle modifications such as smoke cessation and structured exercise are recommended (Jagpal and Navarro-Millan 2018).

1.1.2 Role of inflammatory macrophages in cardiovascular disease and rheumatoid arthritis

Although inflammatory macrophages play an important role in the innate immune system and are required for microbiological defense, the initial inflammatory response can become pathogenic and contribute to disease progression, as it is seen in chronic inflammatory and autoimmune diseases (Sindrilaru et al. 2011), such as arteriosclerosis and RA (Sack et al. 1994, Bories and Leitinger 2017).

The process of atherosclerosis starts with a sterile inflammation triggered by accumulation of cholesterol-rich lipoproteins in subendothelial space of arteries. These lipoproteins get modified, for example, by oxidation or enzymatic cleavage, trigger an inflammatory response and lead to a recruitment of bone marrow-derived monocytes. These monocytes differentiate into macrophages, which take on an inflammatory phenotype in progressing lesions (Moore et al. 2013, Tabas and Lichtman 2017). Inflammatory macrophages change the structure of plaques by releasing proteases and cytokines, and by a defective phagocytic clearance of apoptotic macrophages in advanced plaques (Tabas 2010). In that way macrophages are responsible for plaque necrosis and thinning of the fibrosis cap, which are two crucial properties of a clinically dangerous plaque and can trigger plaque rupture. Macrophages amplify the immune response within the lesion by recruitment of other cells like additional monocytes, neutrophils and T cells (Tabas and Lichtman 2017). Interestingly, a study has shown that inhibition of the proinflammatory cytokine IL-1β by an antibody (Canakinumab) can reduce cardiovascular events in high-risk population (Ridker et al. 2017).

The described inflammation mechanisms in arteriosclerosis display similarities with the chronic inflammation processes of affected joints in RA. Macrophages are a prominent cell component in the inflamed synovium and are mainly responsible for the cytokine-rich proinflammatory environment, which dictates the pathogenesis of RA synovitis (Soler Palacios et al. 2015). These cytokines, including TNF, IL-1β and IL-6, can affect the differentiation of osteoblasts and osteoclasts, which has the potential to lead to bone erosion and joint destruction (Shaw and Gravallese 2016). Interestingly, inflammatory macrophages can amplify tissue inflammation within the synovium by a positive feedback loop maintained by secreted cytokines, such as IL-6, which further promote the development of inflammatory macrophages (Li et al. 2012). Targeting cytokines released by macrophages greatly improved the therapeutic management of RA (Onuora 2017).

In summary, macrophages are a main source for key inflammatory cytokines in RA and CAD and facilitate tissue damage, which leads to joint destruction in RA (Udalova et al. 2016) and plaque destabilization in arteriosclerosis, a major reason for ischemic complications of CAD (Moore and Tabas 2011).

1.2 Immunometabolism

The ability to adapt metabolic processes to different demands of bioenergetic and biosynthetic needs is a basic requirement of all cells. Early studies have described metabolic reprogramming in cancer cells but its implications for immune cells have emerged recently. Immunologists described how alterations in cellular metabolism of immune cells can directly influence their immunological function including cytokine secretion (Kelly and O'Neill 2015, Andrejeva and Rathmell 2017).

Glucose, fatty acid and proteins fuel a variety of closely linked metabolic pathways. The glycolytic pathway, pentose-phosphate pathway, Krebs cycle, fatty acid oxidation and fatty acid synthesis and amino acid metabolism as well as the mitochondrial respiratory chain provide energy and important biosynthetic intermediates. The intermediates are essential for cellular proliferation, the immunological profile of a cell and the modulation of cellular signaling (O'Neill et al. 2016).

Our research focused on the glycolytic pathway and the mitochondrial respiratory chain because it was shown that inflammatory macrophages from CAD patients overutilize glucose to drive cytokine production (Shirai et al. 2016).

These pathways are described in the following chapters in more detail.

1.2.1 Glycolytic pathway

Glycolysis is a cellular process to break down sugar that was taken up by the cell. It plays a central role in energy metabolism by directly producing ATP and additional substrates, which can be used by mitochondria for further energy generation (Pearce and Pearce 2013). Besides its contribution to energy supply, the glycolytic pathway provides the cell with essential metabolic intermediates for the biosynthesis of amino and fatty acids as well as ribose for nucleotide synthesis (O'Neill et al. 2016).

The uptake of glucose from the surrounding of a cell is carried out by transmembrane glucose transporters (GLUTs), which consist of fourteen different isoforms in humans. GLUT1, for instance, is mainly expressed in the brain and in macrophages (Ancey et al. 2018, Koelwyn et al. 2018). After glucose enters the cytosol of the cell, its processing starts through the glycolytic pathway, which degrades one molecule of glucose into two molecules of pyruvate in ten steps with each catalyzed by a specific enzyme (Diskin and Palsson-McDermott 2018) (Figure 1).

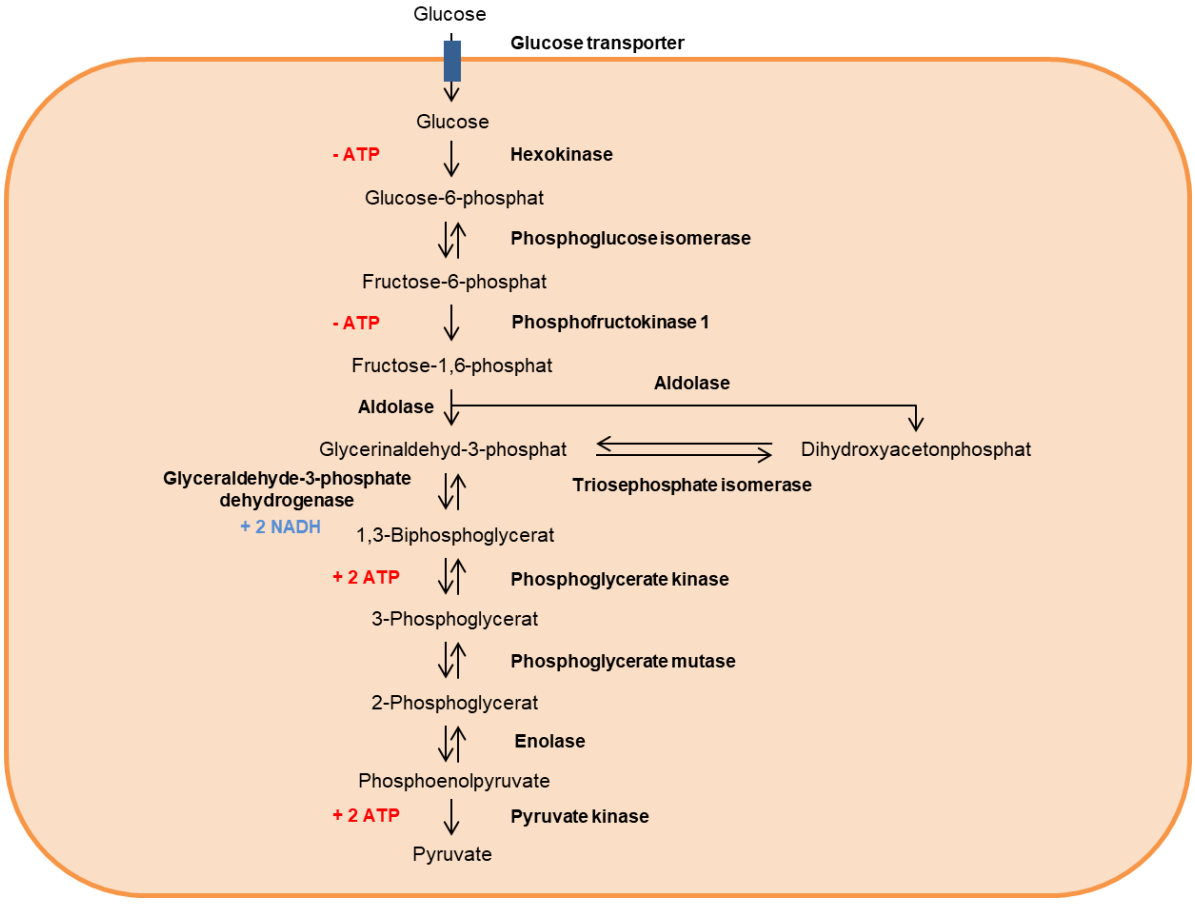


Figure 1: Glucose uptake and utilization

Glucose enters the cell by specific glucose transporters and is then metabolized in multiple steps. One molecule of glucose is processed into two molecules of pyruvate. Each step is catalyzed by a specific enzyme. The reactions of glucose to glucose-6-phosphat and fructose-6-phosphat to fructose-1,6-phosphat consume each one ATP. The transformations of 1,3-biphosphoglycerat to 3-phosphoglycerat as well as phosphoenolpyruvate to pyruvate produce two molecules of ATP. Thus, the total net outcome of these reactions is two ATP molecules. Additionally, two NADH molecules are generated in the reaction of glycerinaldehyd-3-phosphat to 1,3-biphosphoglycerat. Illustration adopted from Li et al. (2015).

Pyruvate generated through glycolysis can either be reduced to lactate by lactate dehydrogenase (LDH) in order to restore NAD^+, which is needed to maintain glycolysis, or it can be oxidized to generate acetyl-coenzyme A. Acetyl-coenzyme A is utilized in the Krebs cycle, to generate NADH and flavin adenine dinucleotide ($FADH_2$), which are then processed by the mitochondrial respiratory chain to produce ATP (Diskin and Palsson-McDermott 2018).

1.2.2 Mitochondrial respiratory chain

The mitochondrial respiratory chain represents the most efficient way of ATP production and consists of large complexes located in the inner membrane of the mitochondria (O'Neill et al. 2016, Diskin and Palsson-McDermott 2018). These complexes utilize NADH and $FADH_2$, generated by the Krebs cycle, and synthesize 32-36 ATPs per molecule of glucose. This is about 15 times more compared to the glycolytic pathway (Uebelhoer and Iruela-Arispe 2016). Besides the generation of ATP, the mitochondrial respiratory chain plays an important role in signaling processes, ignition of cancer and proinflammatory cytokine production through the release of reactive oxygen species (ROS) (Sorbara and Girardin 2011, Sabharwal and Schumacker 2014, Quiros et al. 2016).

1.2.2.1 Structure and function of the mitochondrial respiratory chain

The mitochondrial respiratory chain consists of the electron transport chain (ETC) and the ATP synthase (complex V). The ETC is composed of four different protein complexes (CI-IV) as well as electron carriers cytochrome c and ubiquinone. The ETC accepts electrons form NADH and $FADH_2$ and transfers these electrons through a series of redox reactions from CI to CIV to pump protons from the mitochondrial matrix to the intermembrane space. The result is an electrochemical potential that powers ATP generation at the ATP synthase (Diskin and Palsson-McDermott 2018).

The ETC starts with the oxidation of the electron carrier NADH created through the Krebs cycle at CI (NADH: ubiquinone oxidoreductase), which leads to the transport of four protons in the intermembrane space. CII (succinate dehydrogenase) consists of four subunits of succinate dehydrogenase located within the mitochondrial inner membrane and accepts electrons by oxidizing succinate to fumarate. These electrons from CI and CII are passed to CIII (ubiquinol-cytochrome c oxidoreductase) by the mobile electron carrier ubiquinone (also known as coenzyme Q). Its oxidation at CIII is carried out by a multi-step reaction known as the Q-cycle, which is coupled to the translocation of additional four protons. Cytochrome c is an electron carrier within the intermembrane space and transports electrons to CIV (cytochrome c oxidase), which reduces oxygen to water in order to pump another four protons in the intermembrane space (Berg JM 2002, Sharma et al. 2009, Langston et al. 2017, Diskin and Palsson-McDermott 2018).

In summary, the ETC passes electrons from NADH or $FADH_2$ to molecular oxygen and protons are shifted from the mitochondrial matrix across the mitochondrial inner membrane at CI, CIII and CIV. This leads to a proton-motive force (pmf), which results from the proton electrochemical gradient (ΔpH) and mitochondrial membrane potential gradient (Δψ) (Mitchell 1961, Nicholls 2004). The mitochondrial membrane potential dominates the value of pmf in most conditions and is a crucial factor that indicates the bioenergetic status of the mitochondria (Adam-Vizi and Chinopoulos 2006). In the final step of the mitochondrial respiratory chain this potential is used to generate ATP from ADP and inorganic phosphate at ATP synthase by allowing protons to shift back into the matrix (Mitchell 1961, Nicholls 2004) (Figure 2).

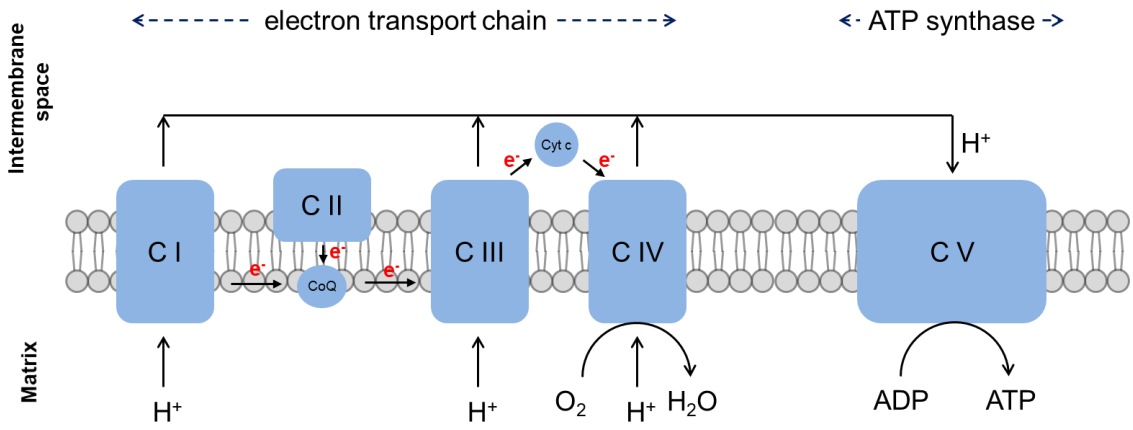


Figure 2: Structure of the mitochondrial respiratory chain

The mitochondrial respiratory chain consists of the electron transport chain (ETC) and the ATP-Synthase. The ETC is composed of four complexes, which passes electrons step by step from high to low redox potentials and protons are shifted from the mitochondrial matrix to the intermembrane space by CI, CIII and CIV. Ubiquinone (also called coenzyme Q) and cytochrome c function as electron carriers within the ETC. The membrane potential created by the translocation of protons is used to synthesize ATP from ADP at the ATP synthase (CV). Illustration adopted from Schon et al. (2012).

1.2.2.2 Source and effects of mitochondrial reactive oxygen species

An inevitable effect of an active mitochondrial respiratory chain is the generation of mitochondrial ROS (mtROS) (Brand 2010). The term ROS comprises different forms of reactive molecules that are created from oxygen such as the superoxide ($O_2^{-\bullet}$), the hydroxyl radical ($OH^{\bullet}$) or hydrogen peroxide (H_2O_2) (Bolisetty and Jaimes 2013). Electrons transferred from one complex to the other can escape from the ETC and reduce oxygen (O_2) to produce $O_2^{\bullet-}$, which is the most intracellular form of mtROS (Murphy 2009, Sena and Chandel 2012).

$O_2^{\bullet-}$ can be transformed to H_2O_2 by the mitochondrial superoxide dismutase or interact directly with nitric oxide ($NO^{\bullet}$) to form peroxynitrite ($ONOO^-$) (Murphy 2009).

In total there are eight electron transfer sites in the mitochondria that have been identified as potential sources of mtROS generation (Sena and Chandel 2012). These sites are CI, CIII, glycerol-3-phosphate dehydrogenase, flavoprotein Q oxidoreductase, cytochrome c, CIV, 2-oxoglutarate and pyruvate dehydrogenase (Brand 2010). Most of these sites are poorly understood but studies have shown that mtROS generation is primarily located at CI and CIII, which have been studied most extensively (Murphy 2009). mtROS generation at the large multi-subunit CI can occur at the flavinmononucleotide, the Fe-S clusters and the coenzyme Q subunits and can result from NADH oxidation or ubiquinone reduction (Brand 2010, Pinegin et al. 2018). In isolated CI, reduced flavin plays the main part of mtROS generation (Kussmaul and Hirst 2006). Three transmembrane subunits of CIII are other important sources of mtROS production. The subunits are cytochrome b, cytochrome c_1 and the Rieske protein, which build the cytochrome bc_1 complex (Bleier and Drose 2013).
It is important to mention, that the described findings of mtROS generating sites have been obtained from *in vitro* experiments and it is unclear at which rate these sites function under physiological conditions in an *in vivo* setting (Brand 2010).
There are several factors, such as mitochondrial membrane potential and activity of ATP generation, which influence the mtROS production (Murphy 2009). Elevated membrane potential, for instance, can lead to higher mtROS generation at CI (Brand 2010).

Formation of mtROS is essential for the functioning of the immune system. mtROS drives proinflammatory cytokine production and is important for defense against bacteria (Angajala et al. 2018). The contribution of mtROS to the activation of the NLRP3 inflammasome, a cytosolic complex that plays a crucial role in innate immunity, drives the production of IL-1β and IL-18 (Chen et al. 2018). Furthermore, the modification of extracellular signal-regulated kinase (ERK), p38 MAPK and c-Jun N-terminal kinase (JNK) by mtROS can also lead to proinflammatory cytokine production (Bulua et al. 2011, Sorbara and Girardin 2011). Additional to its influence on cytokine generation, mtROS is essential for CD4+ T cell activation and can modulate B cell function (Chaudhri et al. 1986, Laniewski and Grayson 2004, Jang et al. 2015).
However, excessive mtROS production can result in the induction of autoinflammatory and autoimmune processes and is associated to several diseases such as diabetes,

neurodegeneration, cancer and inflammatory disorders (Sena and Chandel 2012, van der Burgh and Boes 2015, Lee et al. 2016). In CAD macrophages, ROS induces the redox-sensitive enzyme pyruvatkinase M2 and thereby enhances cytokine production through an activation of the transcription factor signal transducer and activator of transcription 3 (STAT3) (Shirai et al. 2016).

Besides immune cell function, mtROS can alter a variety of other cellular processes including differentiation, autophagy and metabolic adaptation (Sena and Chandel 2012, Dan Dunn et al. 2015).

1.2.3 Aim of the thesis

Cardiovascular comorbidities represent the major cause of death in patients with RA. The risk of myocardial infarction in RA, even at early stages, is higher than that in the general population (England et al. 2016, Mackey et al. 2018). Inflammatory macrophages are key effector cells in both rheumatoid synovitis and the arteriosclerotic plaques of CAD, but an assessment on molecular level to find shared pathogenic mechanisms of these macrophages has not been done. The goal of the thesis was, to study the metabolic phenotype of RA and CAD macrophages, search for shared abnormalities and investigate its implications on this effector cells' function. Dysregulated immunometabolic pathways might represent new therapeutic targets for intervention to decrease CAD risk in RA patients.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Chemical	Source
Macrophage colony-stimulating factor (M-CSF)	eBioscience, USA
Lipopolysaccharid (LPS)	Sigma Aldrich, USA
Phosphate buffered saline (PBS)	BD Biosciences, USA
Trypan blue solution 0.4 %	Merck, Germany
Bambanker	Thermo Fisher Scientific, USA
BenchMark Fetal Bovine Serum (FBS)	Gemini Bio Products, USA

Table 1: Chemicals

2.1.2 Inhibitors and recombinant proteins

Inhibitor/recombinant protein	Source
Interferon-γ (INF-γ)	Sino Biologicals, China
ROS-scavenger TEMPOL	Sigma-Aldrich, USA

Table 2: Inhibitors and recombinant proteins

2.1.3 Auxiliary materials

Material	Source
12-well plate Millicell Cell Culture Inserts	Merck, Germany
96-well plate	Merck, Germany
PCR plates	Thermo Fisher Scientific, USA
Plastic film for PCR plates	Thermo Fisher Scientific, USA

Cellstar polystyrene conical tubes (15, 50 ml)	Thermo Fisher Scientific, USA
Cellstar serological pipette (2, 5, 10, 25 ml)	Thermo Fisher Scientific, USA
Eppendorf reaction tubes	Eppendorf AG, Germany
Eppendorf research pipettes (2.5, 10, 100, 1000 µl)	Eppendorf AG, Germany
Neubauer counting chamber	Sigma-Aldrich, USA
8 cap strip, MicroAmp	Thermo Fisher Scientific, USA
MicroAmp Optical 96-Well Reaction Plate	Thermo Fisher Scientific, USA
PCR reaction tubes, single cap	Thermo Fisher Scientific, USA
Cryotubes	Sigma-Aldrich, USA
Ficoll tubes	BD Biosciences, USA

Table 3: Auxiliary materials

2.1.4 Instruments

Instrument	**Source**
RealPlex2 Mastercycler	Eppendorf, Germany
LSRFortessa	BD Bioscience, USA
GPR Centrifuge	Beckman Coulter, USA
Centrifuge 5804 R	Eppendorf, Germany
LSRii devices	BD Bioscience, USA
Infinite M1000pro	Tecan, Switzerland
FlowJo software	FlowJo LLC, USA
GraphPad Prism V. 7.0 software	GraphPad Prism, USA

Table 4: Instruments

2.1.5 Commercially available systems (kits)

System	Source
Direct-zol RNA MiniPrep	Zymo Research, USA
High-Capacity cDNA Reverse Transcription Kit	Thermo Fisher Scientific, USA
SYBR Green qPCR Master Mix	Thermo Fisher Scientific, USA
Cytofix/Cytoperm Kit	BD Bioscience, USA
ATP Detection Assay Kit	Abcam, England

Table 5: Commercially available systems (kits)

2.1.6 Dyes

Dyes	Source
Tetramethylrhodamine	Life Technologies, USA
Mitotracker Deep Red	Life Technologies, USA
MitoSOX Red	Life Technologies, USA

Table 6: Dyes

2.1.7 Primers

Primer	Source
Glucose Transporter Type 1 forward	5’-TATGTGGAGCAACTGTGTGGT-3’
Glucose Transporter Type 1 reverse	5’-TCCGGCCTTTAGTCTCAGGA-3’
Hexokinase 2 forward	5’-ACGGAGCTCAACCATGACCAA-3’
Hexokinase 2 reverse	5’-AAGATCCAGAGCCAGGAACTC-3’
Lactate dehydrogenase A forward	5’-ATGGCAACTCTAAAGGATCAGC-3’
Lactate dehydrogenase A reverse	5’-ATGGCAACTCTAAAGGATCAGC-3’
NADH dehydrogenase subunit 2 forward	5’-CACCCTTAATTCCATCCACCC-3’

NADH dehydrogenase subunit 2 reverse	5’-TGGGCAAAAAGCCGGTTAG-3’
NADH:ubiquinone oxidoreductase core subunit V1 forward	5’-AGGATGAAGACCGGATTTTCAC-3’
NADH:ubiquinone oxidoreductase core subunit V1 reverse	5’-CAGTCACCTCGACTCAGGGA-3’
Succinate dehydrogenase complex iron sulfur subunit B forward	5’-ACAGCTCCCCGTATCAAGAAA-3’
Succinate dehydrogenase complex iron sulfur subunit B reverse	5’-GCATGATCTTCGGAAGGTCAA-3’
Cytochrome b forward	5’-CCCCACCCCATCCAACAT-3’
Cytochrome b reverse	5’-TCAGGCAGGCGCCAAG-3’
Ubiquinol-cytochrome c reductase core protein I forward	5’-GGGGCACAAGTGCTATTGC-3’
Ubiquinol-cytochrome c reductase core protein I reverse	5’-GTTGTCCAGCAGGCTAACC-3’
Cytochrome c forward	5’CTTTGGGCGGAAGACAGGTC-3’
Cytochrome c reverse	5’-TTATTGGCGGCTGTGTAAGAG-3’
Cytochrome c oxidase subunit 1 forward	5’-TCCGCTACCATAATCATCGCT-3’
Cytochrome c oxidase subunit 1 reverse	5’-CCGTGGAGTGTGGCGAGT-3’
Cytochrome c oxidase subunit 4I1 forward	5’-CAGGGTATTTAGCCTAGTTGGC-3’
Cytochrome c oxidase subunit 4I1 reverse	5’-GCCGATCCATATAAGCTGGGA-3’
ATP synthetase 8 forward	5’-CAACTAAAAATATTAAACACAA ACTACCACC -3’
ATP synthetase 8 reverse	5’-CGTTCATTTTGGTTCTCAGGG-3’
ATP synthase mitochondrial F1 complex assembly factor 1	5’-CAGTGCATCGCCAACCAAG-3’
ATP synthase mitochondrial F1 complex assembly factor 1	5’-TTAAAGGTCTCCACTAACCCGT-3’
Interleukin-6 forward	5’-AGTTCCTGCAGAAAAAGGCAAAG-3’
Interleukin-6 reverse	5’-ATTTGCCGAAGAGCCCTCAG-3’
Interleukin-1β forward	5’-CAGGCTGCTCTGGGATTCTC-3’
Interleukin-1β reverse	5’-GTCCTGGAAGGAGCACTTCAT-3’

Table 7: Primers

2.1.8 Media, buffers and solutions

Media/buffer/solution	Source
RPMI 1640 medium	Thermo Fisher Scientific, USA
glucose-free RPMI 1640	Thermo Fisher Scientific, USA
Lymphprep	STEMCELL Technology, Canada

Table 8: Media, buffers and solutions

2.2 Methods

2.2.1 Patients and controls

The human sample collection included 20 RA and 21 CAD patients as well as 17 age-matched healthy control individuals. RA patients recruited into the study fulfilled the 2010 diagnostic criteria for RA and were positive for anti-CCP antibodies or for rheumatoid factor. The group of CAD patients had at least one documented myocardial infarction and were enrolled more than 90 days after an ischemic event. Demographically matched healthy individuals were recruited from the Stanford Blood Center. They had no personal or family history of autoimmune disease, cancer, chronic viral infection, or any other inflammatory syndrome.

2.2.2 Cells and culture

Peripheral blood mononuclear cells (PBMCs) were extracted from whole blood by density gradient centrifugation using Lymphoprep medium (STEMCELL Technologies). CD14+ monocytes were differentiated into macrophages in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies) supplemented with 20 ng/ml of macrophage colony-stimulation factor (M-CSF) (eBioscience) and 10% of fetal bovine serum (FBS) (Lonza) for 5 days. Macrophages were then stimulated with 100 U/ml of IFN-γ (Sino Biologicals) and 100 ng/ml of lipopolysaccharide (LPS) (Sigma-Aldrich). Inflammatory macrophages were detached from plates using StemPro Accutase Cell Dissociation Reagent (Life Technologies, Thermo Science Fisher).

To scavenge ROS, macrophages were treated with the ROS-scavenger TEMPOL (Sigma-Aldrich).

2.2.3 Quantitative real time-PCR

Total RNA from macrophages was isolated using Direct-zol RNA MiniPrep (Zymo Research). The provided protocol was followed closely with the subsequent exceptions: To elute RNA, we added 35 ul of DNase/RNase-Free Water to the column matrix and centrifuged it at 13.000 g for 90 seconds and repeated this step once. RNA isolation samples were kept at -80°C.

To generate cDNA, reverse transcription was carried out by High-Capacity cDNA Reverse Transcription Kit from Thermo Fisher Scientific. 300 ng RNA were transcribed into cDNA

using a Bio-Rad Thermocycler. After cDNA synthesis, samples were diluted with 80 µl of nuclease free water and stored at -20°C.
The expression of genes was determined using 2×SYBR Green qPCR Master Mix (Biotool.com) and a RealPlex2 Mastercycler (Eppendorf). Gene transcript numbers were determined by normalizing the target gene against a housekeeping gene (β-actin). All measurements were performed in duplicates.

The primers used are listed in Table 7.

2.2.4 Flow cytometry

Quantification of stainings were done using BD LSRFortessa and LSRii devices. Data were analyzed with FlowJo software (Tree Star). For intracellular staining, macrophages were permeabilized using the Cytofix/Cytoperm Kit (BD).

2.2.5 Mitochondrial staining

Mitochondrial membrane potential was analyzed after incubating cells with 50 nM tetramethylrhodamine (TMRM) for 20 minutes at 37°C. For detection of mitochondrial ROS, macrophages were loaded with 5 µM MitoSOX Red for 10 minutes at 37°C. To measure mitochondrial mass, macrophages were stained with Mitotracker Deep Red for 15 minutes at 37°C. Stained macrophages were then washed with PBS and analyzed by flow cytometry. All dyes were purchased from Life Technologies.

2.2.6 Measurement of ATP

After 5 days of in vitro-culture, differentiated macrophages were transferred to 96-well plates with 20.000 cells per well and rested overnight. Cells were then stimulated with with 100 U/ml IFN-γ (Sino Biologicals) and 100 ng/ml of LPS for indicated time and prepared with a luminescent ATP Detection Assay Kit (Abcam) according to the manufacturer protocol. Luminescence was detected using an ‘infinite M1000pro’ plate reader (Tecan).

2.2.7 Statistics

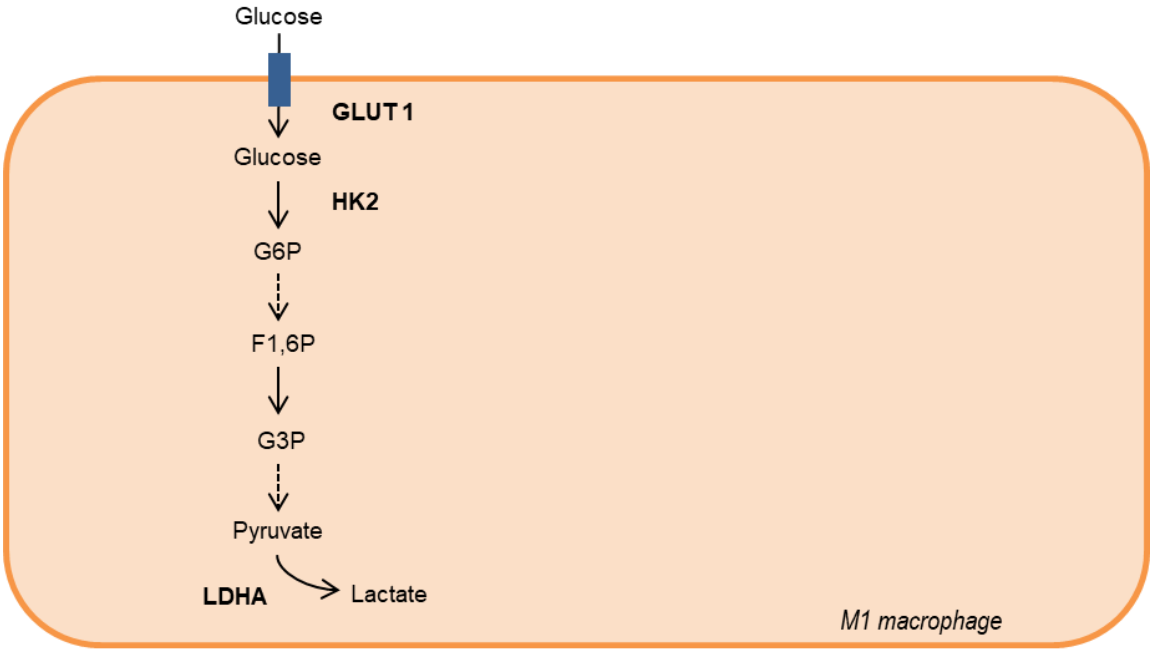
All data are presented as mean plus standard error of the mean (SEM), differences between the groups were tested for their statistical significance by non-parametric unpaired t-test. Paired t-test was applied when analyzing paired data. P-values less than 0.05 were considered significant.

3 Results

The experiments of this thesis consist of two parts. In the first part, the metabolism of macrophages from patients with autoantibody-positive RA and from patients with CAD is analyzed and compared to healthy control (HC) macrophages. In the second part, the immunological effector functions of these macrophages are investigated.

3.1 Increased glycolytic activity in macrophages from RA and CAD patients

Since glucose uptake and utilization is essential for the energy supply and biosynthetic needs of activated macrophages (Kelly and O'Neill 2015), we analyzed genes with a pivotal role in the glycolytic pathway. These genes were *glucose transporter 1* (*GLUT1*), *hexokinase-2 (HK2*) and *lactate dehydrogenase A* (*LDHA*) (Figure 3).



Name	Function
GLUT1 (Glucose transporter 1)	major glucose uptake transporter in macrophages
HK2 (Hexokinase-2)	first enzyme of the glycolytic pathway and rate limiting enzyme
LDHA (Lactate dehydrogenase A)	regenerates NAD^+, that is necessary for maintaining glycolysis

Figure 3: Overview of the glycolytic pathway and investigated genes

After glucose enters the macrophage via GLUT1, its processing starts with the transformation to glucose 6-phosphate (G6P) by HK2. To maintain glycolysis, it is important to regenerate NAD^+ through LDHA.

After stimulating macrophages with LPS and IFN-γ for 24 hours, mRNA levels of *GLUT1* were analyzed by quantitative real-time PCR. Transcript levels of *GLUT1* in macrophages from RA patients were significantly increased ($p < 0.0001$) compared to macrophages from healthy donors. CAD macrophages showed an even more pronounced upregulation of *GLUT1* transcripts ($p = 0.0057$) (Figure 4).

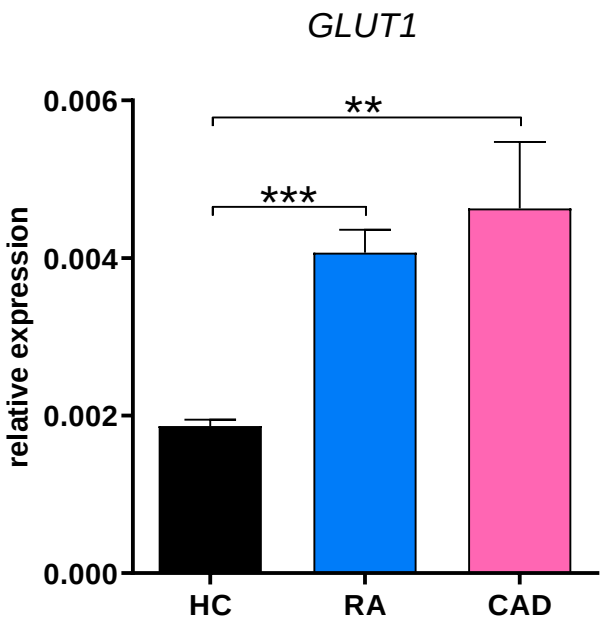


Figure 4: Transcript levels of GLUT1 are higher in RA and CAD macrophages

CD14+ cells isolated from peripheral blood mononuclear cells (PBMCs) of healthy individuals (HC), patients with rheumatoid arthritis (RA) and patients with coronary artery disease (CAD) were differentiated into macrophages using macrophage colony-stimulating factor (M-CSF) and polarized into M1 cells by stimulation with LPS and IFN-γ for 24 hours. GLUT1 mRNA levels were measured using quantitative real-time PCR (n = 4 for all groups). Results are shown as mean ± SEM. Statistical significance was tested by unpaired t-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

In a next step, the gene expression of *HK2* was investigated. In line with high *GLUT1* gene expression of RA and CAD macrophages, *HK2* mRNA levels were significantly increased ($p = 0.009$) in macrophages from RA patients compared to HC. Also macrophages from CAD patients showed higher mRNA levels ($p = 0.0378$) than the healthy control group (Figure 5).

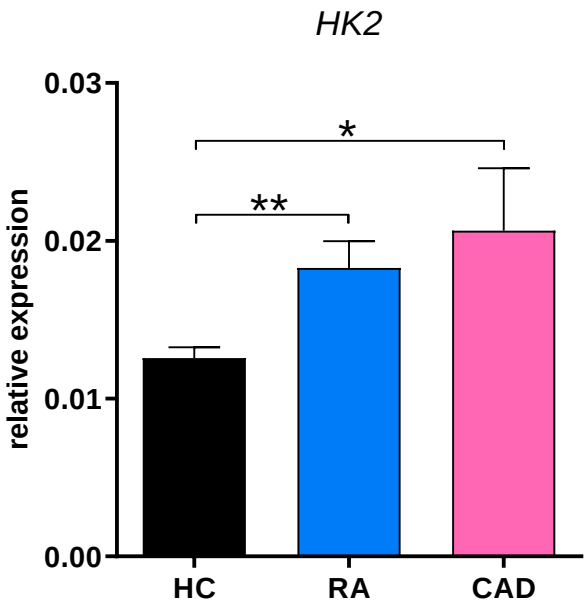


Figure 5: Transcript levels of HK 1 are upregulated in RA and CAD macrophages

Macrophages from HCs, RA patients and, CAD patients were stimulated with LPS/IFN-γ for 24 hours. RNA expression of HK2 genes was determined by quantitative real-time PCR (n = 4 for all groups). Results are shown as mean ± SEM. Significance was determined by unpaired t-test. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Finally, *LDHA* transcript levels showed comparable results as obtained for *GLUT1* and *HK2*. Transcript levels of *LDHA* from patient-derived macrophages were significantly higher compared to HC (RA: p = 0.0184; CAD: p = 0.0004) (Figure 6).

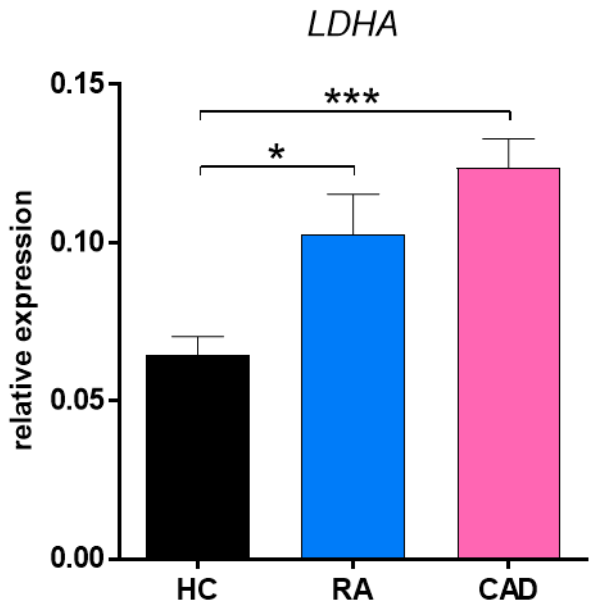


Figure 6: Higher transcript levels of LDHA in RA and CAD macrophages

Monocytes were isolated from four HCs, four RA patients and, four CAD patients, differentiated into macrophages and stimulated with LPS and IFN-γ. 24 hours later LDHA mRNA levels were measured using quantitative real-time PCR. Results are shown as mean ± SEM. Significance was determined by unpaired t-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

In summary, gene expression profiling experiments showed elevated transcript levels of *GLUT1*, *HK2* and *LDHA* in RA and CAD macrophages compared to HC. See figure 7 for a heatmap of the results. This heatmap clearly shows that macrophages from diseased patients overexpress genes required for glucose uptake and processing, which might be indicative for a hypermetabolic state of the cell.

Interestingly, macrophages from CAD patients showed consistently higher transcript levels compared to those from RA patients (Figure 4-6).

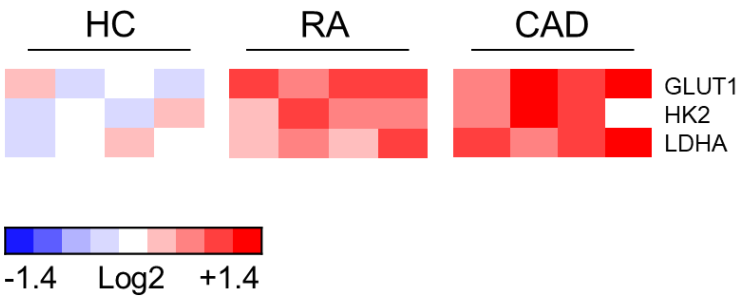


Figure 7: Higher transcript levels of glycolytic genes from RA and CAD macrophages

Macrophages from healthy individuals, patients with RA and, patients with CAD were stimulated with LPS and IFN-γ. 24 hours later mRNA levels were assessed by quantitative real-time PCR (n = 4 for all groups). Heatmap displays the fold change increase of gene expression presented as the log2 value of relative mRNA expression (see color scale). Results are shown as mean ± SEM.

3.2 Hyperactive mitochondria in macrophages from RA and CAD patients

The observation of increased expression of genes important for glucose uptake and breakdown raised the question whether its further processing for energy generation within the mitochondria could be upregulated as well. To address this, we investigated the metabolic status of mitochondria on a transcriptional and functional level.

3.2.1 Upregulated genes of the mitochondrial respiratory chain

The goal of the next experiments was to evaluate the expression of genes encoding for proteins of the mitochondrial respiratory chain, which consists of the electron transport chain (complex I-IV and cytochrome c) and the ATP synthase complex (complex V) (Figure 8) (Diskin and Palsson-McDermott 2018). Since these genes are located within the mitochondrial DNA (mtDNA) as well as the nuclear genome (Quiros et al. 2016), a representative gene of each location was investigated for each complex. In vitro-cultured monocyte-derived macrophages from RA and CAD patients were stimulated with IFN-γ and LPS for 24 hours and mRNA levels were measured by quantitative real-time PCR.

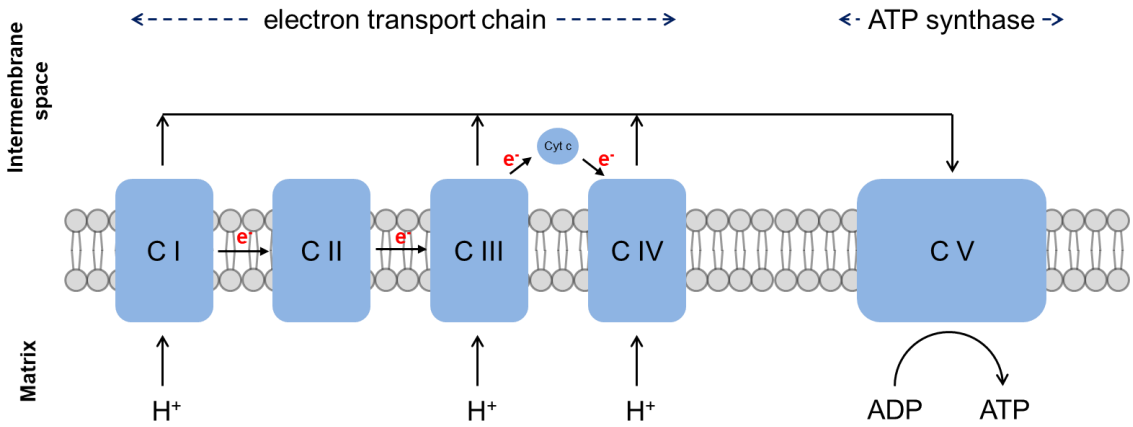


Figure 8: Structure of the mitochondrial respiratory chain

The mitochondrial respiratory chain consists of five complexes with distinct functions and is located in the mitochondrial inner membrane. Complex I-IV are mainly responsible for building up mitochondrial membrane potential by shifting H^+ into the mitochondrial intermembrane space. Complex V uses the membrane potential to generate ATP from ADP. Illustration adopted from Schon et al. (2012).

Electron transport chain

The role of the electron transport chain is to pump H^+ into the mitochondrial intermembrane space, forming an electrochemical proton gradient across the inner mitochondrial membrane needed to generate ATP at complex V (Sharma et al. 2009).

The gene expression profiling of the electron transport chain started with the measurement of mRNA levels of mitochondrial encoded *NADH dehydrogenase subunit 2* (*ND2*) and nuclear encoded *NADH:ubiquinone oxidoreductase core subunit V1* (*NDUFV1*) for complex I. The transcript levels of *ND2* in patient-derived cells were increased compared to HC (RA: $p = 0.056$; CAD: $p = 0.033$) and mRNA levels of *NDUFV1* were significantly increased in macrophages from CAD patients ($p = 0.0008$).

Similar results were obtained for complex II, which functions as an electron carrier between complex I and III and is only encoded by nuclear genes (Scarpulla 2008). The nuclear encoded *succinate dehydrogenase iron-sulfur subunit B* (*SDHB*) was significantly elevated for macrophages from CAD patients compared to HC ($p = 0.0074$).

mRNA levels of mitochondrial encoded c*ytochrome b* (*Cytb*) and nuclear encoded *ubiquinol-cytochrome-c reductase complex core protein 1* (*UQCRC1*) were further investigated for complex III. Compared with healthy controls, patient-derived macrophages showed higher mRNA levels for both genes. mRNA levels of *Cytb* ($p = 0.0119$) and *UQCRC1* ($p = 0.0106$) were markedly increased in macrophages from CAD patients.

Located in the middle of complex III and IV, *cytochrome c* functions as an electron carrier between these complexes (Garrido et al. 2006). The transcription levels of this gene were increased in both patient cohorts compared to HC, with macrophages from CAD patients showing a significant increase of *cytochrome c* ($p = 0.015$).

Finally, complex IV was investigated. Complex IV transports H^+ from the matrix into the intermembrane space by using O_2 (Sharma et al. 2009). To characterize the transcription status of this complex, mRNA levels of mitochondrial encoded *cytochrome c oxidase* (*CO1*) and nuclear encoded *cytochrome c oxidase subunit IV isoform 1* (*COX4I1*) were measured. mRNA level of *CO1* ($p = 0.0053$) and *COX4I1* ($p = 0.0138$) were significantly increased for macrophages from CAD patients compared to HC. mRNA levels of both genes were higher for RA macrophages compared to the healthy control group (*CO1:* $p = 0.16$; *COX4I1:* $p = 0.29$) (Figure 9).

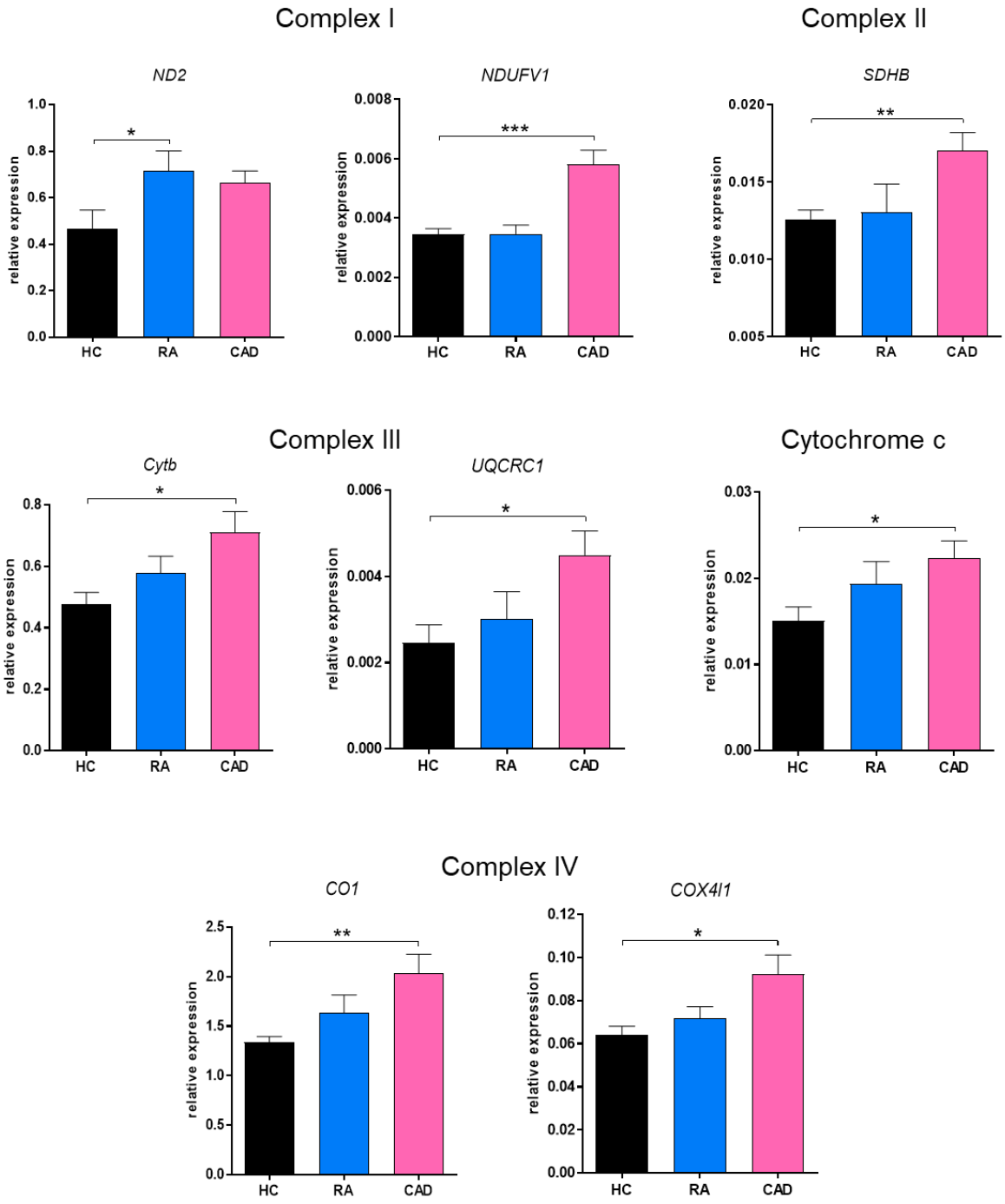


Figure 9: Higher mRNA levels of the electron transport chain in RA and CAD macrophages

Ex vivo-generated macrophages from seven healthy individuals, seven RA as well as seven CAD patients were stimulated with LPS/IFN-γ for 24 hours. mRNA levels were analyzed by quantitative real-time PCR. For complex I, III and IV mitochondrial (left) and nuclear (right) encoded genes were investigated. Complex II and cytochrome c are only encoded by nuclear genes. Results are shown as mean ± SEM. Significance was determined by unpaired t-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

ATP synthase

Complex V, also known as ATP synthase complex, produces ATP via oxidative phosphorylation by using the inner membrane potential (Sharma et al. 2009). To address the question whether increased gene expression of complex I-IV and cytochrome c go along with higher mRNA levels of the ATP synthase complex, mRNA levels of mitochondrial encoded *ATP synthase protein 8* (*Atpase8*) and nuclear encoded *ATP synthase* mitochondrial *F1* (*ATPaf1*) complex were measured. *ATPase8* mRNA levels of macrophages from RA patients were significantly increased ($p = 0.044$) compared to those from healthy individuals. An even stronger upregulation was observed in macrophages from CAD patients ($p = 0.0088$). *ATPaf1* mRNA levels were significantly increased for macrophages from CAD patients ($p = 0.0025$) (Figure 10).

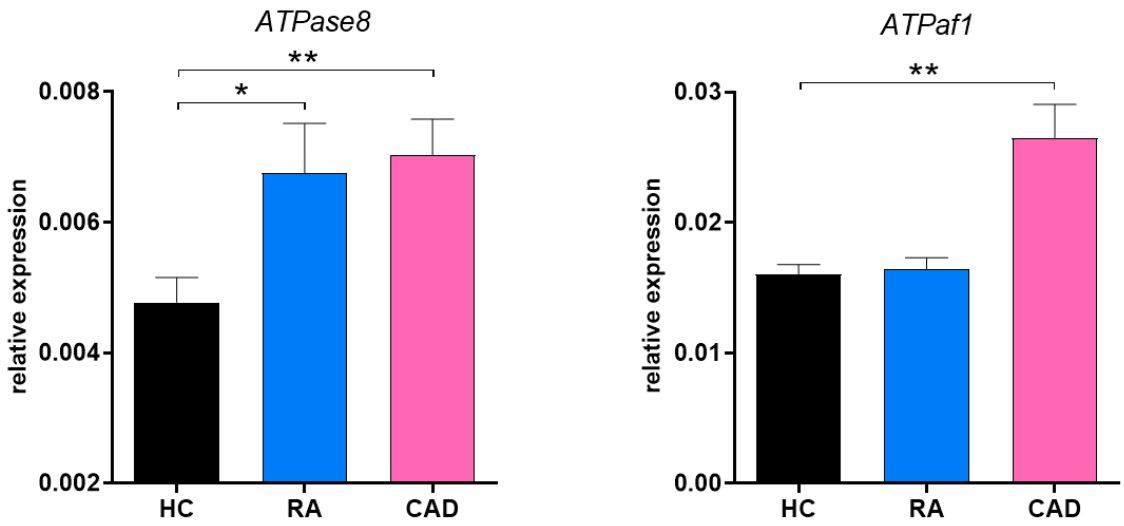


Figure 10: Upregulated transcript levels of complex V in RA and CAD macrophages

Macrophages from HC, RA patients as well as CAD patients were stimulated for 24 hours with LPS and IFN-γ. Bar graphs represent averaged data of gene expression measured by quantitative real-time PCR (n = 7 for all groups). Results are shown as mean ± SEM. Significance was determined by unpaired t-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Summary

Nuclear and mitochondrial encoded gene transcripts of the electron transport chain and the ATP synthase complex were upregulated in RA and CAD macrophages compared to HC macrophages. Additionally, our data revealed the following pattern: transcript levels of macrophages from RA patients were increased compared to HC. CAD macrophages showed an even higher elevation in transcript levels than those from RA patients (Figure 11).

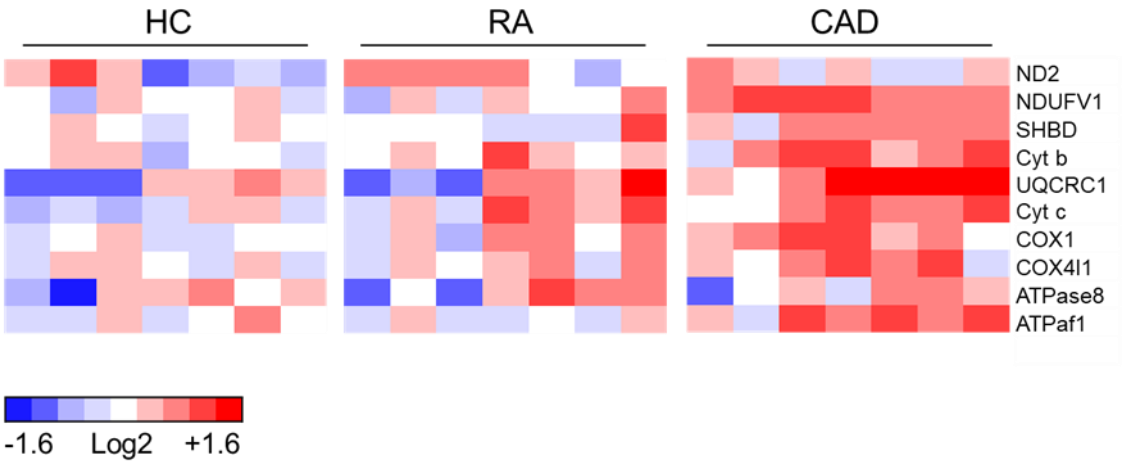


Figure 11: Increased mRNA levels of nuclear and mitochondrial encoded genes of the mitochondrial respiratory chain

CD14+ cells isolated from PBMCs of healthy individuals, patients with RA and, patients with CAD were differentiated into macrophages and stimulated with LPS and IFN-γ. 24 hours later mRNA levels were assessed by quantitative real-time PCR (n = 7 for all groups). Heatmap displays the fold change increase of gene expression presented as the log2 value of relative mRNA expression (see color scale). Results are shown as mean ± SEM.

The observed upregulated transcriptional levels of genes encoding proteins of the mitochondrial respiratory chain are indicative for an enhanced mitochondrial biogenesis in RA and CAD macrophages. To further validate this observation, mitochondrial mass, a surrogate marker for increased mitochondrial biogenesis, was quantified by flow cytometry in the next experiment. In line with the previous results, mitochondrial mass was increased by macrophages from RA patients ($p = 0.1226$) and by those from CAD patients ($p = 0.0006$) (Figure 12).

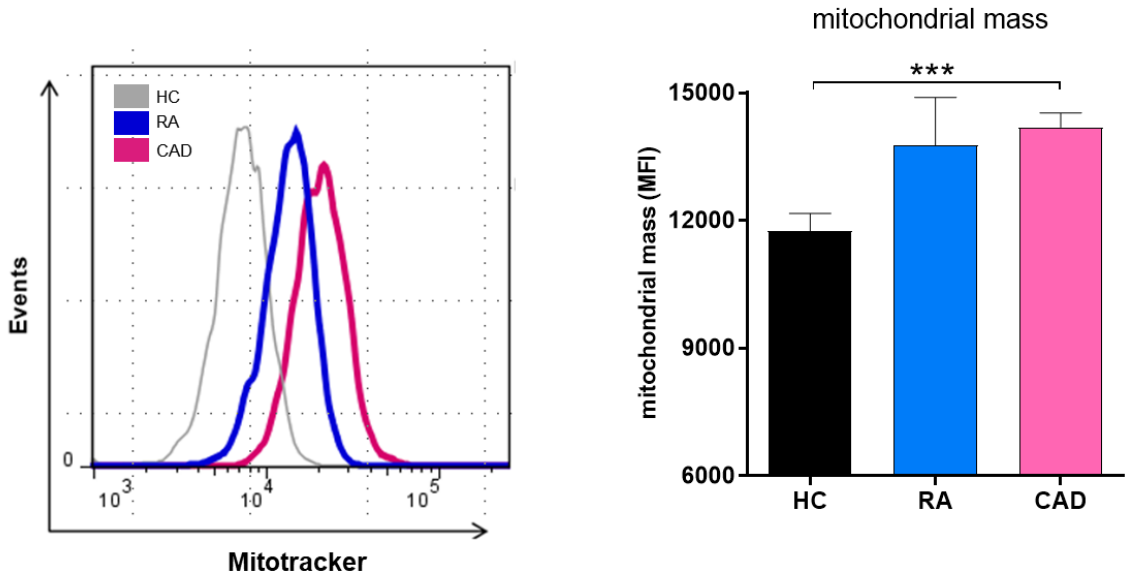


Figure 12: Elevated mitochondrial mass for RA and CAD patients

Monocytes were isolated from healthy individuals, RA patients and, CAD patients and differentiated into macrophages using M-CSF. After stimulation with LPS and IFN-γ for 24 hours, mitochondrial mass was measured by flow cytometry of Mitotracker Deep Red. Representative histograms and MFIs from 6 samples in each group are shown. Results are shown as mean ± SEM. Significance was determined by unpaired t-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

3.2.2 Increased mitochondrial activity in RA and CAD macrophages

To test whether increased transcription of the mitochondrial respiratory chain and higher mitochondrial mass impact the function of mitochondria, its membrane potential and ATP production were measured in macrophages from RA and CAD patients.

In line with high gene transcripts of the mitochondrial electron transport chain (*complex I-IV* and *cytochrome c*), which pumps protons into the intermembrane space (Diskin and Palsson-McDermott 2018), mitochondrial membrane potential was significantly higher in RA and CAD macrophages compared to HC macrophages. Macrophages from RA patients showed a remarkable increase ($p < 0.0001$) compared to HC macrophages. Even higher membrane potential was detected in cells from CAD patients ($p = 0.0028$) (Figure 13) (Zeisbrich et al. 2018).

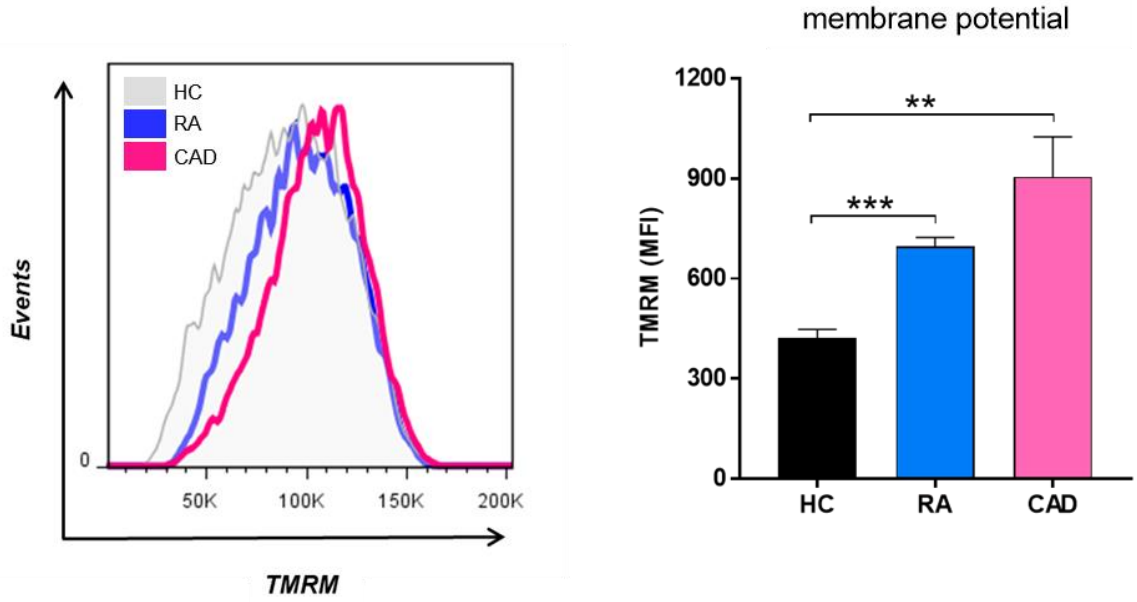


Figure 13: Increased mitochondrial membrane potential in RA and CAD patients

Mononuclear cells isolated from controls, patients with RA and, patients with CAD were differentiated into macrophages and stimulated with LPS and IFN-γ for 24 hours. Mitochondrial membrane potential was assessed by flow cytometry with TMRM staining. Representative histograms and MFIs from 6 samples in each group are shown. Results are shown as mean ± SEM. Illustration adopted from Zeisbrich et al. (2018). Significance was determined by unpaired t-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

After quantifying mitochondrial membrane potential, we measured ATP production of macrophages by using a luminescence assay. ATP production of macrophages from RA patients was significantly elevated (p = 0.0014) compared to control. The result for CAD macrophages was again higher than for RA macrophages (p = 0.0052) (Figure 14) (Zeisbrich et al. 2018).

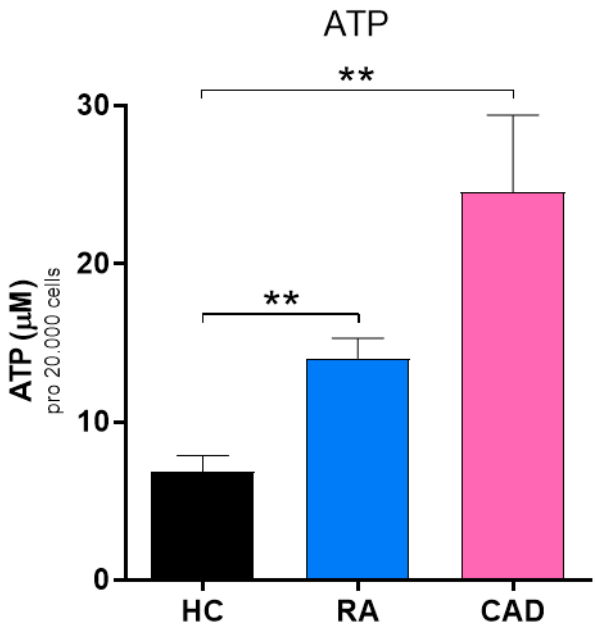


Figure 14: Increased ATP production in RA and CAD patients

Macrophages from patients with RA, patients with CAD, and healthy controls were stimulated with LPS and IFN-γ. Intracellular ATP concentrations per 20.000 activated macrophages measured in 6 samples each group was determined by a luminescent reaction. Results are shown as mean ± SEM. Significance was determined by unpaired t-test. Illustration adopted from Zeisbrich et al. (2018). *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Together, these data demonstrate a hyperactive metabolic state in patient-derived macrophages expressed by an upregulation of glycolytic enzymes and the mitochondrial respiratory chain on transcriptional level as well as a significant increase in mitochondrial mass, membrane potential and ATP production (Figure 15). Throughout our experiments, cells from RA patients had an intermediate phenotype, outperformed by cells from CAD patients.

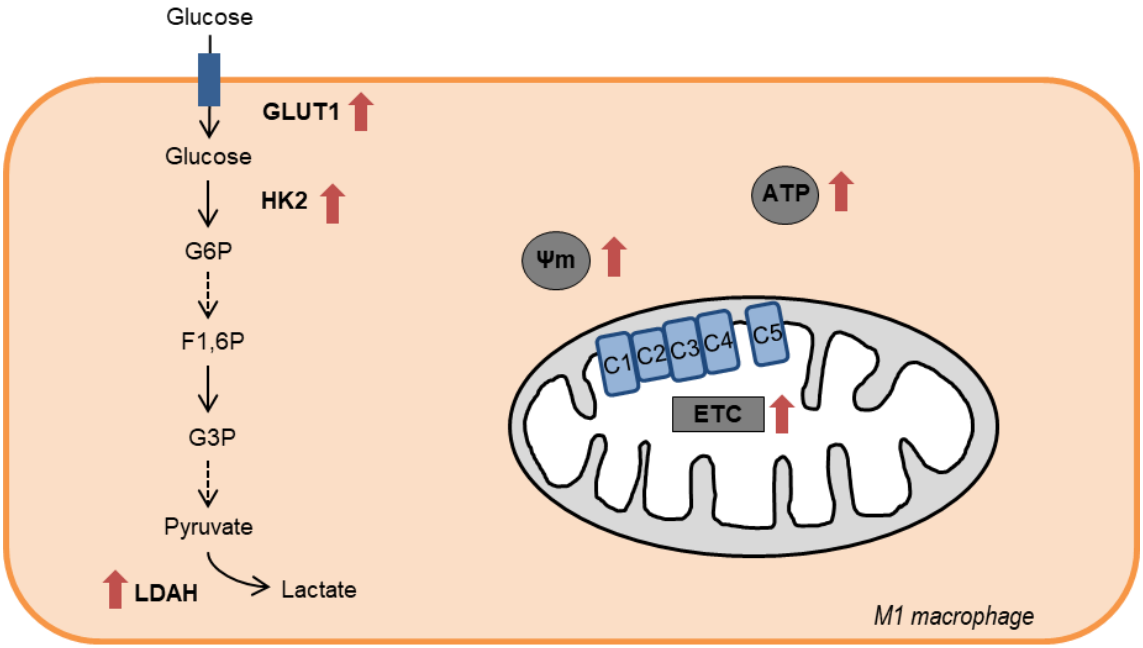


Figure 15: Hyperactive state of macrophages from RA and CAD patients

Patient-derived macrophages show upregulated glycolytic activity and mitochondrial electron transport chain as well as mitochondrial mass and membrane potential, which leads to an increased generation of ATP.

3.3 Increased ROS and cytokine production in RA and CAD macrophages

Former studies showed that metabolic activity is related to reactive oxygen species (ROS) production (Korge et al. 2008). To quantify mitochondrial ROS production in hypermetabolic macrophages of RA and CAD patients, we stained macrophages with MitoSox Red fluorescent, which is representative for mitochondrial ROS production. The data showed that RA and CAD mitochondria generated significantly larger amounts of mitochondrial ROS as an inevitable byproduct of high mitochondrial activity (RA: p = 0.0001; CAD: p = 0.0009) (Figure 16) (Zeisbrich et al. 2018).

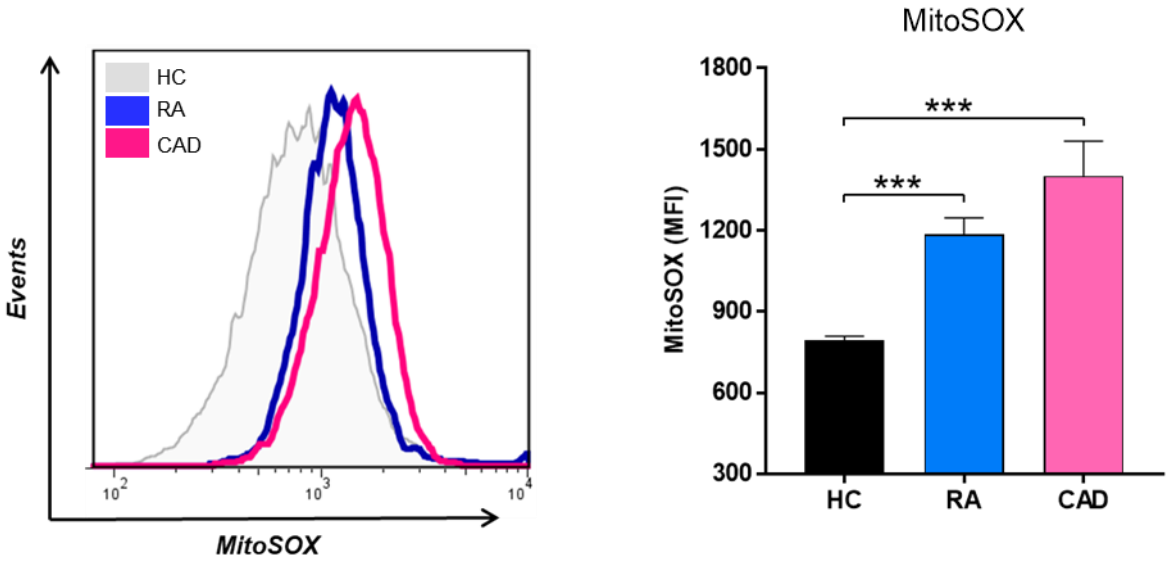


Figure 16: Hypermetabolic state leads to higher ROS production in RA and CAD patients

Mitochondrial ROS was quantified by flow cytometric analysis of MitoSOX Red of activated macrophages derived from healthy donors (HC), RA as well as CAD patients. Representative histograms and MFIs from 6 samples in each group are shown. Results are shown as mean ± SEM. Illustration adopted from Zeisbrich et al. (2018). Significance was determined by unpaired t-test. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Mitochondrial ROS functions as a signaling molecule, which can lead to an increase of proinflammatory cytokine production through different pathways (Naik and Dixit 2011, Wang et al. 2014). The proinflammatory cytokines IL-1β and IL-6 have been directly implicated as disease drivers in RA and CAD patients (Bradham et al. 2014) and were analyzed in the following experiments.

First, gene expression levels of IL-1β were measured by quantitative real-time PCR. Compared with healthy controls, RA macrophages generated significantly (p = 0.0302) higher number of IL-1β transcripts. CAD macrophages showed even higher gene expression levels of IL-1β (p = 0.0031) (Figure 17).

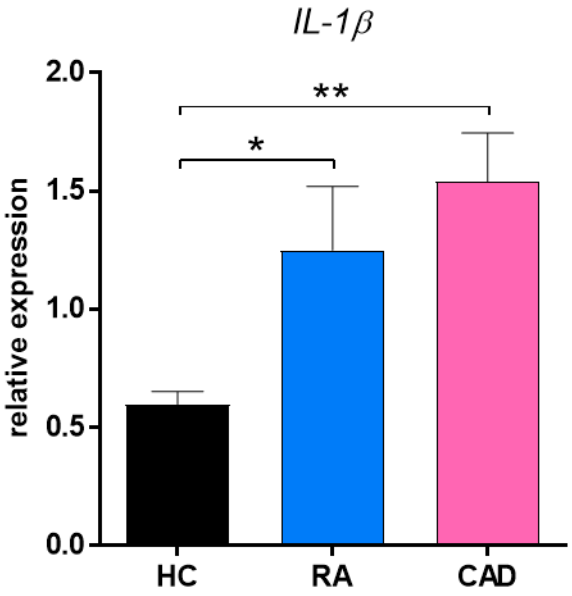


Figure 17: RA and CAD macrophages produce excessive amounts of IL-1β

Monocytes were isolated from healthy controls, RA and CAD patients, then differentiated into macrophages using M-CSF and stimulated with LPS and IFN-γ for 6 hours. Gene expression of IL-1β was measured by quantitative real-time PCR (n = 5 for all groups). Results are shown as mean ± SEM. Significance was determined by unpaired t-test. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Next, the gene expression level of proinflammatory cytokine IL-6, which is now considered as a reliable biomarker of RA and CAD (Ridker et al. 2000, Prasad et al. 2015) was studied. The experiment showed that RA and CAD macrophages produced excessive amounts of IL-6 compared to HC (RA: p = 0.0045; CAD: p = 0.024) (Figure 18).

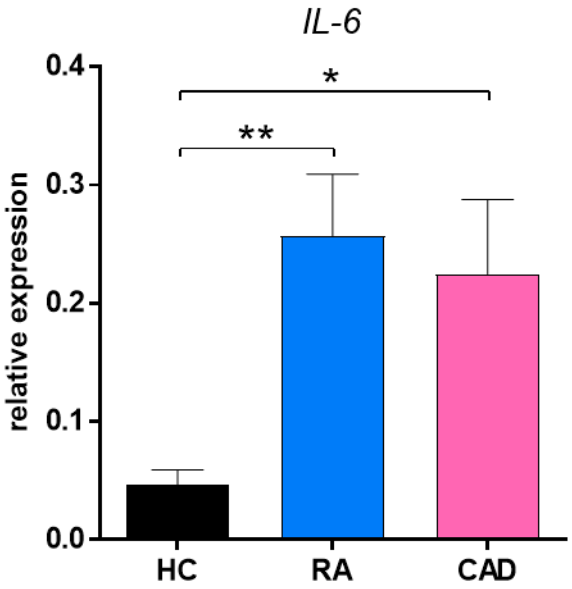


Figure 18: RA and CAD macrophages produce excessive amounts of IL-6

Macrophages from healthy individuals, RA and CAD patients were differentiated and stimulated as described above. Transcript levels of IL-6 were assessed by quantitative real-time PCR (n = 5 for all groups). Results are shown as mean ± SEM. Significance was determined by unpaired t-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

In summary, increased expressions of ROS and proinflammatory genes (IL-1β and IL-6) were observed in macrophages from patients with RA and CAD compared to those of healthy individuals (Figure 19).

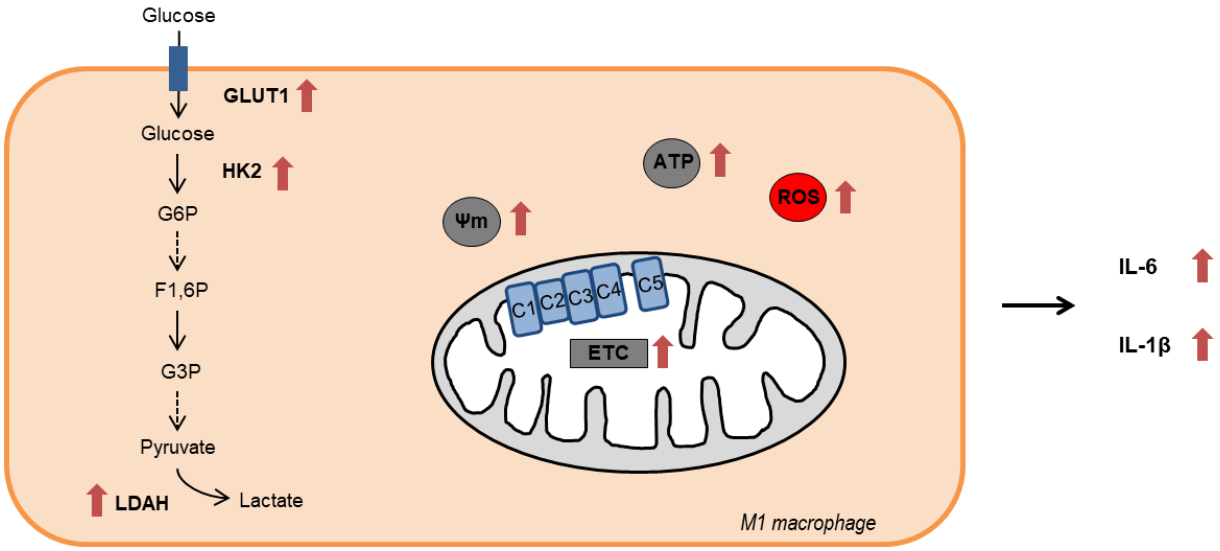


Figure 19: Higher ROS and cytokine production in RA and CAD macrophages

Macrophages from RA and CAD patients showed upregulated ROS, IL-1β and IL-6 levels compared to HC.

3.4 Elevated IL-6 and IL-1β expression in RA and CAD macrophages is ROS dependent

To test whether there is a connection between increased ROS levels and the excessive cytokine expression in RA and CAD patients, the gene transcript levels of IL-1β and IL-6 were analyzed in the presence or absence of a ROS scavenger. Scavenging superoxide with the ROS-Inhibitor Tempol significantly decreased the expression of IL-1β for RA patients ($p = 0.001$) and CAD patients ($p = 0.0008$) (Figure 20).

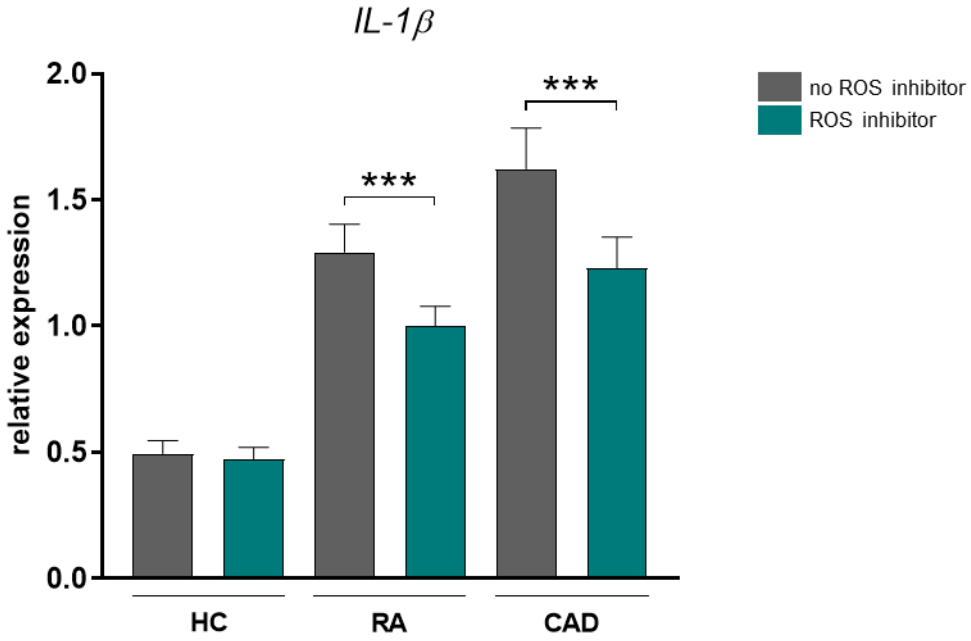


Figure 20: ROS removal reduces IL-1β genes of RA and CAD macrophages

Macrophages were stimulated for 6 hours with LPS and IFN-γ in the absence (grey) and presence (green) of the ROS scavenger Tempol (50 µM). mRNA levels of IL-1β were analyzed by quantitative real-time PCR. Results are shown as mean ± SEM. Unpaired t-test was applied. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

To provide additional evidence for the association of ROS with levels of cytokine production, we analyzed IL-6 mRNA levels before and after ROS removal.

In accordance with the data obtained for IL-1β, IL-6 transcription levels were significantly reduced for RA and CAD patients after the removal of ROS (RA: $p = 0.002$; CAD: $p = 0.0066$) (Figure 21).

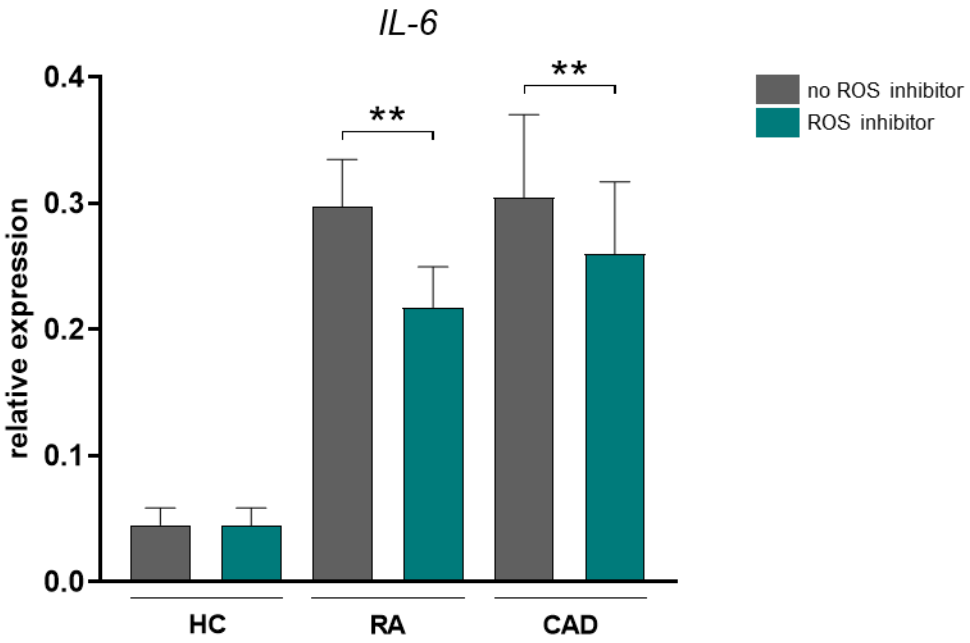


Figure 21: ROS removal reduces IL-6 genes of RA and CAD macrophages

Macrophages from healthy individuals, RA patients and, CAD patients were stimulated for 6 hours under M1-polarizing conditions in the absence (grey) and presence (green) of the ROS scavenger Tempol (50 μM). Gene expression of IL-6 was measured by quantitative real-time PCR. Results are shown as mean ± SEM. Unpaired t-test was applied. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

These data show that eliminating ROS corrects increased cytokine production of hyperinflammatory RA and CAD macrophages.

4 Discussion

The risk for myocardial infarction in patients with RA is increased by 2-fold compared to the general population (Peters et al. 2009) and cardiovascular disease represents the leading cause of mortality in these patients (Maradit-Kremers et al. 2005). This finding cannot be explained by traditional risk factors, such as smoking, hypertension, diabetes mellitus, and hyperlipidemia (Crowson et al. 2013). However, atherosclerosis that underlies coronary artery disease was recently recognized as a chronic inflammatory condition and it seems likely that there is an association between the inflammatory status of RA and the vascular inflammation in atherosclerosis (Hansson and Hermansson 2011, Bories and Leitinger 2017). The most abundant cell type in atherosclerotic plaques or rheumatoid joints are inflammatory macrophages, they are considered to be a potential cellular link for the cardiovascular risk of RA patients (Sack et al. 1994). Activated macrophages facilitate tissue damage, which leads to joint destruction in RA (Udalova et al. 2016) and atherosclerotic plaque destabilization in CAD patients (Moore and Tabas 2011).

Despite the assumption that inflammatory macrophages could drive cardiovascular disease in patients with chronic rheumatic conditions, an assessment on the molecular level has not yet been done. In recent years, metabolic reprogramming of macrophages and its implications for inflammatory cell function have become an emerging field (Tannahill et al. 2013, Everts et al. 2014, Mills and O'Neill 2016). In this thesis, a metabolic and immunological analysis of RA and CAD macrophages demonstrates how metabolic changes in innate immune cells change an effector cell phenotype.

The metabolic assessment started with transcriptional profiling of genes important for glucose uptake and processing in inflammatory macrophages from RA and CAD patients. Patient-derived macrophages upregulated pivotal parts of the glycolytic machinery, including glucose transporter GLUT1 and the enzymes HK2 and LDAH, indicative for fundamental abnormalities in glucose utilization.

These results are in line with other studies, which have shown that glycolysis is an important metabolic pathway for macrophages with a pro-inflammatory phenotype, for example, by supporting the secretory activity of these cells (Cramer et al. 2003, Ryan and O'Neill 2017). Another demonstration of the importance of glycolysis for immune cell function was that inhibition of glycolysis with 2-deoxyglucose (inhibits hexokinase) blocked macrophage

activation in vitro and suppressed inflammation in mouse macrophages (Michl et al. 1976, Hamilton et al. 1986). Enhanced glycolysis does not only occur in inflammatory macrophages, but also in other immune cells such as activated effector T cells (Michalek et al. 2011), activated B cells (Doughty et al. 2006) and activated natural killer cells (Donnelly et al. 2014). Therefore, upregulated glycolysis can be considered an important metabolic change in most immune cells. However, glycolysis may not only be associated with inflammatory cell functions since human regulatory T cells need glycolysis for its suppressive function (De Rosa et al. 2015).
Interestingly, cancer cells show similarities in the metabolic reprograming compared to inflammatory macrophages by increasing glycolysis following mutations of oncogenes or tumor suppressor genes (Andrejeva and Rathmell 2017). The crucial question is, why all these cells - including RA and CAD macrophages - upregulate glycolysis, which is considered a relatively inefficient way of producing ATP compared to the alternative fate of glucose via oxidation in the Krebs cycle and transfer of electrons into the electron transport chain, which generates about 15 times as much molecules of ATP per molecule of glucose (Locasale and Cantley 2010). The answer is that glycolysis might provide certain advantages to the cell. First, glycolysis is a rapid way to generate ATP and enables the cell to adapt quickly to changes in bioenergetic needs (O'Neill et al. 2016). The increased rate of ATP production resulting from glycolysis presents a selective growth advantage (Gatenby and Gillies 2004) and is required during the process of toll-like receptor (TLR) driven activation (Everts et al. 2014). Second, high glycolytic rates benefit the anabolic need of activated cells through the production of several glycolytic intermediates (Gatenby and Gillies 2004, Everts et al. 2014, Yu et al. 2016), which are integrated into different metabolic pathways with sufficient substrates remaining to generate Ac-CoA and drive the Krebs cycle (Deberardinis et al. 2008). Finally, glycolysis creates cofactors of enzymatic reactions such as NADH (Everts et al. 2014). Altogether, enhanced glycolysis ensures a rich and rapid supply of substrates to meet the bioenergetic and biosynthetic needs of an immune cell and enables the cell to carry out its effector function in a flexible way.

The glycolytic pathway in RA and CAD macrophages was analyzed under aerobic conditions in an *in vitro* setting. Aerobic glycolysis was initially described in cancer cells (Warburg effect) with a defect in mitochondrial respiration, but is now considered to also happen in cells with a correct functioning electron transport chain (Stefano and Kream 2015, Goetzman and Prochownik 2018). To gain more information about the fate of the mitochondria in RA and

CAD macrophages, we studied the mitochondrial status on transcriptional and functional level as a next step of our metabolic assessment.

The mitochondrial respiratory chain consists of five different complexes with distinct functions (Diskin and Palsson-McDermott 2018). The increased expression of genes encoding for proteins in complexes I, III and IV is indicative for a higher proton translocation across the inner mitochondrial membrane and was confirmed by measuring an upregulated mitochondrial membrane potential in RA and CAD macrophages. In line with its elevation and higher transcript levels of the ATP synthase complex (complex V), ATP was significantly increased in patient-derived macrophages.

Taken together, we observed a hyperactive metabolism in macrophages from RA and CAD patients where glycolysis as well as mitochondrial activity are synergistically enhanced. This metabolic phenotype also known as hybrid metabolism, is also described in rapidly proliferating normal and cancer cells, where upregulated aerobic glycolysis is used to feed the Krebs cycle and increase mitochondrial respiration to provide the most efficient ATP production and biomass accumulation (LeBleu et al. 2014, Viale et al. 2015, Agnihotri and Zadeh 2016, Goetzman and Prochownik 2018). In breast cancer and glioma cells, for instance, 80 % of ATP production is oxidative and 20 % glycolytic (Martin et al. 1998, Guppy et al. 2002, Pasdois et al. 2003). In contrast, in RA patients, it has been shown that CD4 T cells are energy-deprived with glucose hypometabolism and low ATP production (Yang et al. 2013), which is exactly the opposite of the investigated metabolic program of inflammatory macrophages. This observation is crucial for the development of therapies with the goal of changing dysfunctional metabolic pathways as a treatment for these diseases. For instance, a restriction of glucose intake could be a therapy for the observed hypermetabolic state of RA and CAD macrophages but might worsen the situation for hypometabolic CD4 T cells in RA patients.

However, the increased capacity of RA and CAD macrophages to generate more ATP led to a significant elevation of mtROS production. mtROS comes as an inevitable byproduct of high mitochondrial activity (Sabharwal and Schumacker 2014), where complex I and III represent a major source of ROS production (Murphy 2009). mtROS can function as a signaling molecule and influences various cellular processes (Quiros et al. 2016), including proinflammatory cytokine production through the activation of the NLRP3 inflammasome (Sorbara and Girardin 2011) or the modification of extracellular signal-regulated kinase (ERK), p38 MAPK and c-Jun N-terminal kinase (JNK) (Bulua et al. 2011). Besides the positive effect of mtROS on proinflammatory gene expression, it is a potent driver of metabolic reprogramming that results

in enhanced reliance on glycolysis (Sena and Chandel 2012). Investigations on molecular level revealed that ROS induces the redox-sensitive enzyme pyruvatkinase M2 (PKM2 - catalyzes the final step of glycolysis) and thereby enhances the glycolytic flux as well as cytokine production via an activation of the transcription factor signal transducer and activator of transcription 3 (STAT3) in CAD macrophages (Shirai et al. 2016). Consistent with these studies, our results demonstrate that macrophages from RA and CAD patients were in a hyperinflammatory state and generated significantly higher IL-1β and IL-6 mRNA levels than cells from the healthy control group. Scavenging mitochondrial ROS had anti-inflammatory effects by decreasing IL-1β and IL-6 mRNA levels compared to those of healthy individuals, confirming our hypothesis of ROS dependent production of IL-1β and IL-6. However, using ROS scavengers as therapy of CAD patients has not been successful so far, because of low efficacy and relevant adverse effects (Kornfeld et al. 2015). Rewiring the metabolic program could be a new promising therapeutic approach to decrease mitochondrial ROS in patient-derived macrophages, prevent inflammation in plaques and reduce atherosclerotic burden in RA and CAD patients.

In summary our data show that monocyte-derived macrophages from patients with RA or CAD share a common metabolic phenotype of upregulated glycolysis and mitochondrial activity. This energy-rich phenotype leads to an increased generation of mtROS, which is responsible for excess production of IL-1β and IL-6. An interesting finding of this thesis was that throughout our experiments, we observed a clear hierarchy with CAD macrophages showing higher glycolysis, mitochondrial activity and ATP production, greater increase in ROS generation and higher cytokine production than RA macrophages. This hierarchy might represent substantial risk of myocardial infarction in RA patients, but a higher risk in CAD patients. The risk of cardiovascular events in RA patients may rise with an increase of metabolic activity in RA macrophages. Further investigations are required to connect the hypermetabolic state in RA macrophages and future cardiovascular events.

A limitation of this thesis is the observation of monocyte-derived macrophages in an *in vitro* setting. That way, we did not take the actual environment of these macrophages into account: the atherosclerotic plaque for CAD patients and the synovial lesion of RA joints. An important aspect is how the tissue-specific microenvironmental cues shape the metabolic and inflammatory phenotype of these macrophages. The function of macrophages depends on several interacting variables and factors within the microenvironment (Gosselin et al. 2014).

Macrophages interact and regulate the functional activity of other cells, such as T cells, with whom they share the tissue site (Janeway CA Jr 2001, Wynn et al. 2013). Considering the complex interaction between innate and adaptive effector cells in chronic inflammatory processes, the macrophage contribution to the progression of arteriosclerosis are likely be aggravated by effector T cell responses. Other factors within the microenvironment, for instance in atherosclerotic plaques, are cholesterol crystals, oxidized lipids, and danger-associated molecular patterns from dying cells (Moore et al. 2013). A more comprehensive evaluation will be necessary to determine how these macrophages prioritize these multiple stimuli and adopt a specific metabolic phenotype and to understand the interplay of different cell types in each tissue environment.

Another limitation is, that we have not looked at metabolic intermediates created within the Krebs cycle. Studies report about a broken Krebs cycle in activated macrophages where different metabolites, notably, citrate, succinate and fumarate accumulate (Infantino et al. 2011, Tannahill et al. 2013, Arts et al. 2016). These intermediates can contain a wide spectrum of different effects and influence the function of macrophages in various ways (Mills and O'Neill 2016). Citrate, for instance, is shuttled to the cytosol, where it is used for prostaglandin generation (Ricciotti and FitzGerald 2011).

The observed hypermetabolic phenotype of RA and CAD macrophages, which enables cells to generate large amounts of ATP, mtROS, IL-1β and IL-6, is most likely a result of interlinked and co-regulated processes. In further experiments Glycogen synthase kinase 3b (GSK3b) - a critical regulator of cellular metabolism and mitochondrial activation (Sieber et al. 2016, Jellusova et al. 2017) - was identified as a key regulator in reprogramming macrophages from RA and CAD patients (Zeisbrich et al. 2018). In most cells, including macrophages, GSK3b is constitutively active (Park et al. 2011), and can be inactivated by phosphorylation (Cross et al. 1995). It was shown that inactivation of GSK3b leads to mitochondrial hyperactivation and gene induction of *GLUT1* in macrophages of RA and CAD patients (Zeisbrich et al. 2018). In future investigations it will be necessary to study upstream kinases of GSK3b, which enhances the activity of the respiratory chain by phosphorylating GSK3b. An interesting candidate to investigate represents AMP-activated protein kinase (AMPK). AMPK phosphorylates GSK3b (Zheng et al. 2015) and is also a sensor of cellular energy status (Brunmair et al. 2004, Konrad et al. 2005).

Data presented in this thesis have implications for the understanding and the management of RA, atherosclerosis, and the increased cardiovascular risk in RA patients. Macrophages of patients with metabolic abnormalities such as heightened extracellular glucose concentrations as part of type 1 or type 2 diabetes mellitus, might have higher glycolytic and thus greater pro-inflammatory and pro-atherosclerotic activity, which may relate to the acceleration of atherosclerosis in these patients.

Our investigation demonstrates a shared hypermetabolic phenotype of macrophages form RA and CAD patients, which drives the immunological output of these cells. Molecular mechanisms were shown that will serve as a first step to explain the progression of atherosclerosis in RA patients and represent a foundation for the development of new interventions for RA and CAD patients to alter the metabolic phenotype of these macrophages.

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List of Abbreviation

Ac-CoA	acetyl coenzyme A
ACR	American College of Rheumatology
AMPK	AMP-activated protein kinase
ATPaf1	ATP synthase *mitochondrial* F1
Atpase8	ATP synthase protein 8
CAD	coronary artery disease
CCP	cyclic citrullinated peptides
CI-V	complex I-V
CO1	cytochrome c oxidase
COX4I1	cytochrome c oxidase subunit IV isoform 1
CRP	c-reactive protein
CVD	cardiovascular disease
Cytb	cytochrome b
Cytc	cytochrome c
DMARD	disease-modifying antirheumatic drugs
ERK	signal-regulated kinase
ETC	electron transport chain
EULAR	European League Against Rheumatism
$FADH_2$	flavin adenine dinucleotide
FBS	fetal bovine serum
GLUT1	glucose transporter 1
GSK3b	glycogen synthase kinase 3b
HC	healthy control
HK2	hexokinase-2
INF-γ	Interferon-γ
JNK	c-Jun N-terminal kinase
LDHA	lactate dehydrogenase A
LPS	lipopolysaccharide

MCP	metacarpophalangeal
M-CSF	macrophage colony-stimulating factor
MI	myocardial infarction
MTP	metatarsophalangeal
mtROS	mitochondrial reactive oxygen species
MTX	methotrexate
NADH	nicotinamide adenine dinucleotide
ND2	NADH dehydrogenase subunit 2
NDUFV1	NADH:ubiquinone oxidoreductase core subunit V1
NTP	nucleoside triphosphate
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PIP	proximal interphalangeal
Pmf	proton-motive force
RA	rheumatoid arthritis
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
SDHB	succinate dehydrogenase iron-sulfur subunit B
SEM	standard error of the mean
STAT3	signal transducer and activator of transcription 3
TLR	toll-like receptor
TMRM	tetramethylrhodamine
TNF	tumor necrosis factor
UQCRC1	ubiquinol-cytochrome-c reductase complex core protein 1

List of Publications

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Declaration of Authorship

Ich versichere, dass ich die Arbeit ohne fremde Hilfe und ohne Benutzung anderer als der angegebenen Quellen angefertigt habe und dass die Arbeit in gleicher oder ähnlicher Form noch keiner Prüfungsbehörde vorgelegen hat. Alle Ausführungen, die wörtlich oder sinngemäß übernommen wurden, sind als solche gekennzeichnet.

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