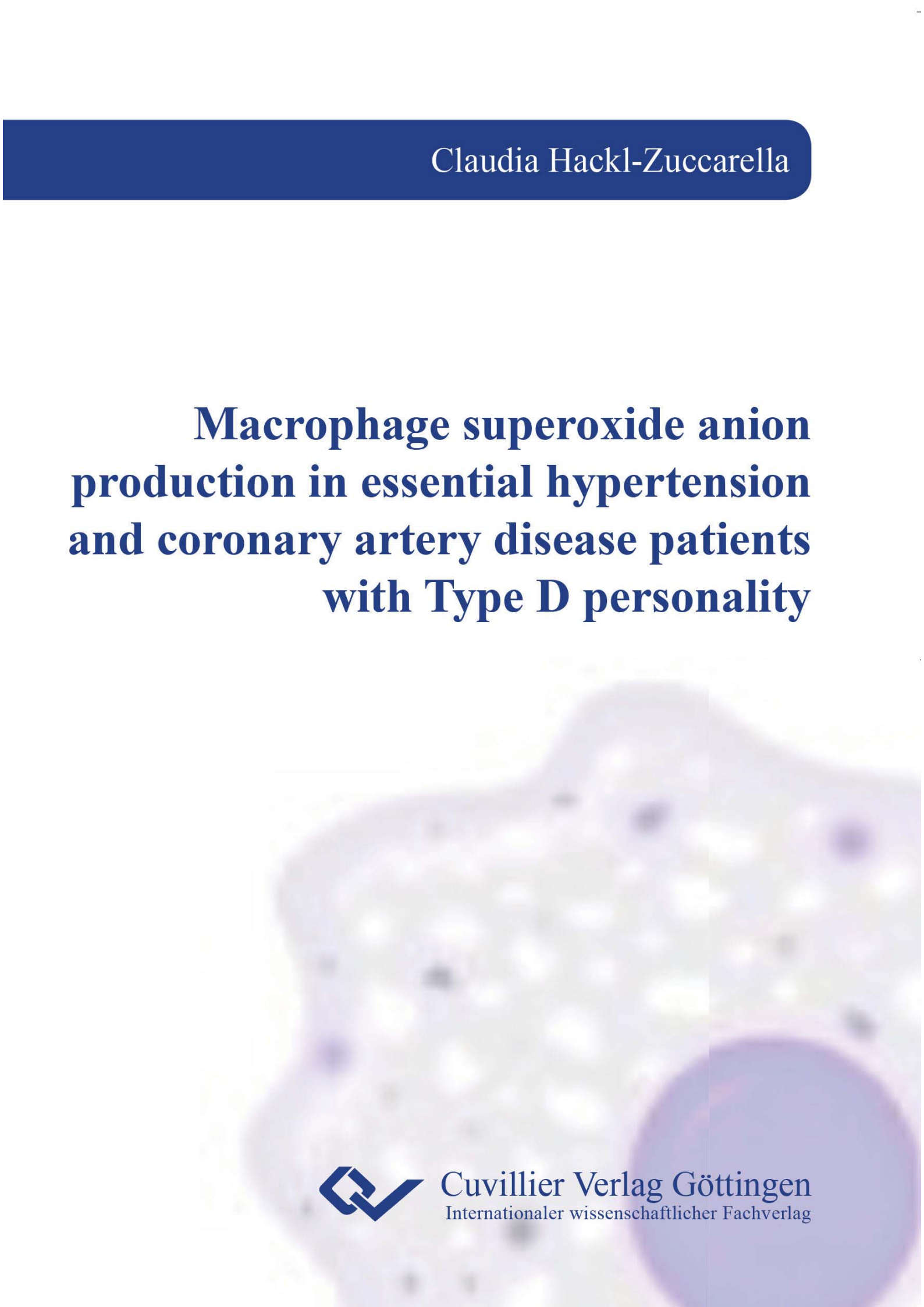
Claudia Hackl-Zuccarella

Macrophage superoxide anion production in essential hypertension and coronary artery disease patients with Type D personality



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To my beloved husband *Christopher Hackl*

ABSTRACT

Essential hypertension is a major risk factor for atherosclerosis and related coronary artery disease, whereas Type D personality is suggested to constitute an independent psychosocial risk factor for poor cardiac prognosis in coronary artery disease patients. However, the mechanisms underlying these associations remain unclear. Both inflammatory macrophages and superoxide anions have been proposed to play a pivotal role in the pathogenesis of atherosclerosis, as the underlying pathophysiological process of coronary artery disease.

This investigation aimed to examine superoxide anions released by human inflammatory macrophages in individuals with essential hypertension and coronary artery disease patients with Type D personality, in order to shed more light on the mechanisms that link hypertension with an increased risk of atherosclerosis and Type D personality with poor cardiac prognosis in coronary artery disease patients, respectively. Therefore, in a first step, a simple and accurate *in vitro* method for the measurement of superoxide anions by human inflammatory macrophages was implemented and validated.

Superoxide anion production by human inflammatory macrophages, measured by means of the reliable and valid *in vitro* WST-1 assay, was higher both in individuals with essential hypertension and in coronary artery disease patients with Type D personality compared to their control groups. Furthermore, mean arterial blood pressure as well as the two Type D subscales ‘negative affectivity’ and ‘social inhibition’, and their interaction, were associated with higher macrophage superoxide anion production.

These findings suggest that superoxide anion production by inflammatory macrophages may play a mechanistic role in the mediation of both atherosclerotic risk in hypertension and poor cardiac prognosis in coronary artery disease patients with Type D personality.

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CONTENTS

TABLES

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ABBREVIATIONS

2K-1C = 2-kidney 1-clip

A

ACE = Angiotensin-converting enzyme

ACS = Acute coronary syndrome

Ang II = Angiotensin II

ANS = Autonomic nervous system

$ApoE^{-/-}$ = Apolipoprotein E-deficient mice

B

BDI = Beck Depression Inventory

BMI = Body mass index

BP = Blood pressure

C

CAD = Coronary artery disease

CCA = Common carotid artery

CCL2 = C-C motif chemokine ligand 2

CCL5 = C-C motif chemokine ligand 5

CGD = Chronic granulomatous disease

CHF = Chronic heart failure

CRP = C-reactive protein

CSSS = Chronic Stress Screening Scale

CVD = Cardiovascular disease

D

DAG = Diacylglycerol

DASH = Dietary Approaches to Stop Hypertension

DBP = Diastolic blood pressure

DNA = Deoxyribonucleic acid

DCA = Directional coronary atherectomy

DOCA = Deoxycorticosterone acetate
DS14 = Type D Scale-14
DS16 = Type D Scale-16
Duox = Dual oxidases

E

EAS = European Atherosclerosis Society
EPQ = Eysenck Personality Questionnaire
ESC = European Society of Cardiology
ESH = European Society of Hypertension

F

FAD = Flavin adenine dinucleotide
FMLP = Formylmethionine-leucyl-phenylalanine
FSS = Functional social support

H

HDL = High-density lipoprotein
HPA axis = Hypothalamic-pituitary-adrenal axis
HPPQ = Heart Patients Psychological Questionnaire
HR = Heart rate
HRV = Heart rate variability
HSC = Hematopoietic stem cell
Hsp = heat shock protein

I

IGF = Insulin-like growth factor
IL = Interleukin
IMT = Intima-media thickness
IFN-γ = Interferon-gamma

K

kg = Kilogram

L

LDL = Low-density lipoprotein
LPS = Lipopolysaccharide
LTB4 = Leukotriene B4
LVEF = Left ventricular ejection fraction
LVH = Left ventricular hypertrophy

M

M1 = Classically activated macrophages
M2 = Alternatively activated macrophages
MAP = Mean arterial blood pressure
MerTK = Mer receptor tyrosine kinase
MHC = Major histocompatibility complex
MI = Myocardial infarction
mm Hg = Millimeters of mercury
MPS = Mononuclear phagocyte system
mRNA = Messenger RNA

N

NA = Negative affectivity
NADPH = Nicotinamide adenine dinucleotide phosphate
NEO-FFI = NEO Five-Factor Inventory
NK cells = Natural killer cells
NO = Nitric oxide
NOS = Nitric oxide synthase
Nox = NADPH oxidase
NSTEMI = non-ST-segment elevation acute myocardial infarction

P

PI3-K = Phosphatidylinositol 3-kinase

PAF = Platelet-activating factor
PAC = p21-activated kinase
PBMCs = Peripheral blood mononuclear cells
phox = Phagocyte oxidase
PKC = Protein kinase C
PMA = Phorbol myristate acetate
PNS = Parasympathetic nervous system

R

RNS = Reactive nitrogen species
ROS = Reactive oxygen species

S

SBP = Systolic blood pressure
SBN/y = Sabra hypertension-resistant rats
SBH/y = Sabra hypertension-prone rats
SHR = Spontaneously hypertensive rats
SI = Social inhibition
SNS = Sympathetic nervous system
SSS = Structural social support
STAI = State-Trait Anxiety Inventory
STEMI = ST-segment elevation acute myocardial infarction

T

TABP = Type A behavior pattern
TBBP = Type B behavior pattern
Type D = Type D personality
TGFß = Transforming growth factor beta
Th1 = T-helper 1 lymphocytes
Th2 = T-helper 2 lymphocytes
TLR4 = Toll-like receptor 4
TNF-α = Tumor necrosis factor-alpha

U

UAP = Unstable angina pectoris

V

VE = Vital exhaustion

VSMCs = Vascular smooth muscle cells

W

WHHR = Watanabe heritable hyperlipidemic rabbits

WHO = World Health Organisation

WST-1 = 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-dis-ulfophenyl)-2H tetrazolium, monosodium salt

1 INTRODUCTION

Essential hypertension – defined as a chronic medical condition characterized by a sustained elevation of blood pressure (BP) in the arteries of unknown cause (Mancia et al., 2013, Messerli, Williams & Ritz, 2007; Nandhini, 2014) – is a major risk factor for atherosclerosis and related coronary artery disease (CAD; Alexander, 1995; Hajjar, Kotchen & Kotchen, 2006; Mancia et al., 2013; Perk et al., 2012). Type D personality (Type D) – characterized by a combined expression of high negative affectivity (NA) and high social inhibition (SI; Denollet, Sys & Brutsaert, 1995) – is suggested to be an independent psychosocial risk factor for poor cardiac prognosis in CAD patients (Denollet, Sys, Stroobant, Rombouts, Gillebert & Brutsaert, 1996; Denollet, 2000; Denollet, Pedersen, Ong, Erdman, Serruys & van Domburg, 2006a; Denollet, Pedersen, Vrints & Conraads, 2006b). However, the mechanisms that link hypertension with increased risk of atherosclerosis, and thus CAD and Type D with poor cardiac prognosis in CAD patients, respectively, are not fully understood.

Atherosclerosis, as the underlying process of CAD, is defined as a progressive chronic inflammatory process of arterial wall thickening, and is characterized by intense immunological activity (Hansson & Libby, 2006). The innate immune system plays a pivotal role in the initiation and progression of the inflammatory process in atherosclerosis, with inflammatory macrophages (i.e. tissue-based phagocytic immune cells) derived from circulating peripheral blood monocytes (Davies, Jenkins, Allen & Taylor, 2013; Epelman, Lavine & Randolph, 2014) being key cells in this process (Ghattas, Griffiths, Devitt, Lip & Shantsila, 2013; Ley, Miller & Hedrick, 2011; Moore & Tabas, 2011). A key innate immune effector function of inflammatory macrophages, in particular classically activated (M1) inflammatory macrophages, is microbicidal activity, i.e. the killing of microbes (Colin, Chinetti-Gbaguidi & Staels, 2014; Mosser & Edwards, 2008; Zhang & Wang, 2014). Microbicidal activity, in turn, is largely due to the production of reactive oxygen species (ROS), particularly superoxide anions (de Oliveira-Junior, Bustamente, Newburger & Condino-Neto, 2011; Halliwell, 2006), derived from the activated multisubunit enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase located in the phagolysosomal and plasma membrane of macrophages (De Oliveira-Junior, 2011; Cathcart, 2004).

The emphasis on the inflammatory nature of atherosclerosis raised the possibility to examine new immunological pathways in order to understand the relationship between hypertension and atherosclerosis as well as Type D and poor cardiac prognosis in CAD. Indeed, research in hypertension and CAD indicated that NADPH oxidase-derived superoxide anions likely play a crucial role in the pathogenesis and progression of atherosclerosis, and thus CAD. However, in these studies, NADPH oxidase-derived superoxide anion production was analyzed either in vascular wall cells (Guzik et al., 2000a; Guzik et al., 2006; Vendrov, Hakim, Madamanchi, Rojas, Madamanchi & Runge, 2007) or in circulating macrophage precursor cells (Fortuño, Oliván, Beloqui, San José, Moreno & Diez, 2004; Moreno, San José, Fortuño, Beloqui, Diez & Zalba, 2006; Moreno et al., 2014; Watanabe, Yasunari, Nakamura & Maeda, 2006). Therefore, the possible contribution of NADPH oxidase-derived superoxide anion production by inflammatory M1 macrophages to atherosclerosis in hypertension and CAD is unclear.
Although no studies have examined the role of NADPH oxidase-derived superoxide anion production in CAD patients with Type D, there is evidence for a dysregulation of the immune system as a potential underlying mechanism that may explain the association between Type D and poor cardiac prognosis in CAD. Studies in patients with chronic heart failure (CHF) have shown that Type D independently predicts increased circulating levels of tumor necrosis factor-alpha (TNF-α) and TNF-α soluble receptors (Conraads, Denollet, De Clerck, Stevens, Bridts & Vrints, 2006; Denollet, Conraads, Brutsaert, De Clerck, Stevens & Vrints, 2003). Notably, TNF-α is a proinflammatory cytokine that has been shown to be involved in atherosclerosis by inducing the production of ROS (Zhang et al., 2009). Furthermore, Type D has been associated with increased oxidative stress (Kupper, Gidron, Winter & Denollet, 2009) and endothelial dysfunction (van Dooren et al., 2016), where superoxide anions play an important role. It should be emphasized that endothelial dysfunction accelerates atherosclerosis and has been shown to be an independent predictor of poor cardiac prognosis in CAD patients (Halcox et al., 2002; Heitzer, Schlinzig, Krohn, Meinertz & Münzel, 2001; Suwaidi, Hamasaki, Higano, Nishimura, Holmes & Lerman, 2000).

Given the importance of NADPH oxidase-derived superoxide anion production in essential hypertension as well as superoxide anions and Type D in CAD, we aimed to investigate NADPH oxidase-derived superoxide anion production by human inflammatory M1 macrophages – as pivotal inflammatory cells in the atherosclerotic process – in individuals with essential hypertension and CAD patients with Type D, in order to provide new insights into the potential mechanisms that link both hypertension with increased risk of

atherosclerosis and Type D with poor cardiac prognosis in CAD. Before addressing these research questions, our first aim was to implement and validate an appropriate method for the measurement of NADPH oxidase-derived superoxide anion production by human inflammatory M1 macrophages.

The presentation of the thesis is organized as follows: The theoretical background briefly reviews the main research areas of macrophages, essential hypertension, CAD with its underlying process of atherosclerosis, and Type D. In all research areas, a specific emphasis is placed on the role of NADPH oxidase-derived superoxide anions. Following this, summaries of the empirical studies and their results are presented. The detailed manuscripts of the studies are provided in the Appendix. Finally, the thesis concludes with a general discussion of the results and directions for future research.

2 THEORETICAL BACKGROUND

The purpose of this chapter is to review the main research areas and their association with NADPH oxidase-derived superoxide anion production in order to understand the nature of our empirical studies reported in chapter 4.

This chapter includes five main sections: Section 2.1 provides an understanding of macrophages; section 2.2 presents the cardiovascular risk factor essential hypertension with an emphasis on the role of NADPH oxidase-derived superoxide anions; section 2.3 includes CAD and the underlying process of atherosclerosis with an emphasis on the role of macrophages and NADPH oxidase-derived superoxide anion production in this process. Section 2.4 describes Type D as a psychosocial risk factor for poor cardiac prognosis in CAD patients and elucidates potential biological mechanisms underlying this association, while section 2.5 briefly summarizes the theoretical background.

2.1 Macrophages

Macrophages are tissue-based immune cells (i.e. white blood cells or leukocytes) and were first discovered by the Russian zoologist Ilya Illyich Mechnikov in the late 19th century (Davies et al., 2013; Epelman et al., 2014), who described their phagocytic nature (Nathan, 2008; Tauber, 2003). Macrophages are present in lymphoid and non-lymphoid tissues of the body (Geissmann, Manz, Jung, Sieweke, Merad & Ley, 2010) and are known to play an essential role in both the innate and the adaptive immune system (Abbas, Lichtman & Pillai, 2007; Biswas, Chittezhath, Shalova & Lim, 2012; Dale, Boxer & Liles, 2008).

Macrophages are multi-functional cells and exhibit functional diversity based on their microenvironment (Biswas et al., 2012; Haldar & Murphy, 2014). Beside their hallmark function of phagocytic ability in order to engulf and kill microbes, tumor cells, and other invaders, macrophages also have the ability to present antigen to T lymphocytes, and thus activate specific defense mechanisms of the adaptive immune system (Adams, 1994; Woods, 2000). Furthermore, macrophages are potent secretory cells (Adams, 1994). The secreting molecular products are involved in inflammation, growth regulation, and hematopoiesis. In addition, macrophages influence lymphocyte function, affect tissue repair, act as autoregulatory factors, or are microbicidal (Woods, 2000). This functional diversity of macrophages is first and foremost required to maintain homeostasis (Gordon & Taylor, 2005;

Wynn, Chawla & Pollard, 2013). Figure 1 provides an overview of the major effector functions of macrophages.

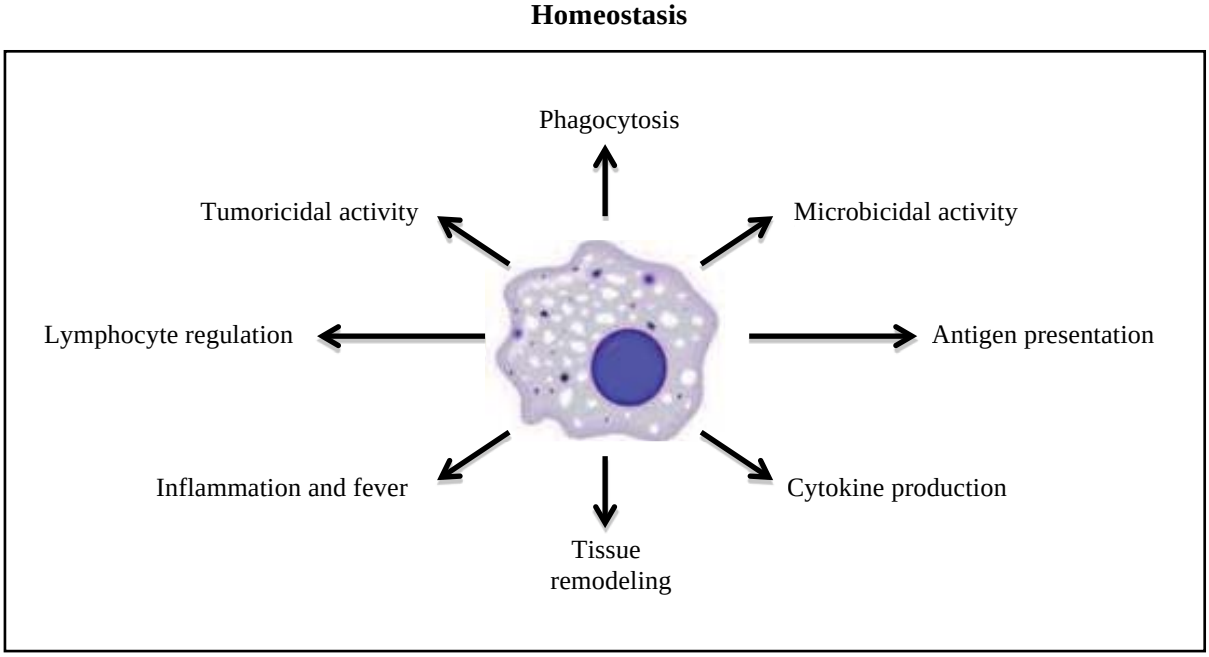

Figure 1: Major effector functions of macrophages. Adapted from Woods (2000).

2.1.1 Origin of macrophages

Macrophages are mononuclear phagocytes and thus cells of the *mononuclear phagocyte system* (MPS). Van Furth and colleagues first introduced the concept of the MPS in the late 1960s and early 1970s (van Furth, Cohn, Hirsch, Humphrey, Spector & Langevoort, 1972). This system is defined as a hematopoietic cell lineage derived from progenitor cells in the bone marrow, the so-called hematopoietic stem cell (HSC; Cao, Harris & Wang, 2015; Hume, 2006; van Furth et al., 1972), and encompasses cells with similar morphology, origin, and biology including promonocytes and their precursors in the bone marrow, circulating blood monocytes, tissue macrophages, and dendritic cells (Hume, 2008; van Furth et al., 1972).

The differentiation of macrophages in the MPS is described as follows (see Figure 2): HSC as progenitor cells give rise to monoblasts, promonocytes, and finally monocytes, which enter into the peripheral blood, where they circulate for several days. Afterwards, monocytes migrate from the blood into tissues, where they further mature into tissue-specific macrophages of the bone (osteoclast), lung (alveolar macrophages), central nervous system (microglial cells), connective tissue (histiocytes), gastrointestinal tract, liver (Kupffer cells),

spleen, and peritoneum (Abbas et al., 2007; Mosser & Edwards, 2008). Notably, tissue-specific macrophages are continually present in the tissue. Both the development of monocytic cells (i.e. monoblast, promonocyte, and monocyte) and the differentiation of monocytes into macrophages are driven by a protein called macrophage colony-stimulating factor (Mosser & Edwards, 2008; Takahashi, 2001). It should be emphasized that macrophages are differentiated from circulating blood monocytes under non-inflammatory and inflammatory conditions (Takahashi, 2001).

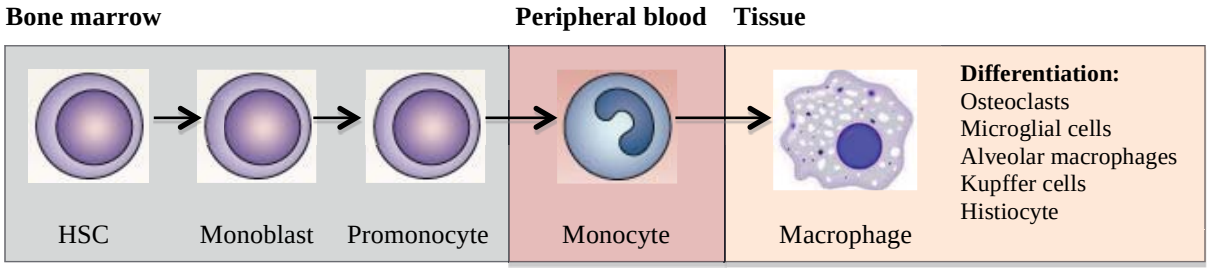

Figure 2: Mononuclear phagocyte system. Adapted from Mosser & Edwards (2008).

2.1.2 Classification of macrophages under inflammatory conditions

Inflammatory macrophages derived from circulating peripheral blood monocytes represent key cells in inflammatory processes (Duffield, 2003; Gordon & Taylor, 2005) and are termed as monocyte-derived inflammatory macrophages (Italiani & Boraschi, 2014). In contrast to tissue-specific macrophages, inflammatory macrophages are only present in the inflamed tissue under inflammatory conditions (Abbas et al., 2007; Mosser & Edwards, 2008).

During inflammation, the migration of circulating monocytes from the bloodstream into inflamed tissue is extremely enhanced and leads to an increase of macrophages at inflamed tissue sites (Mosser & Edwards, 2008) in order to optimize the immune defensive firepower (Italiani & Boraschi, 2014). To increase their competence for host defense under inflammatory conditions, monocyte-derived inflammatory macrophages additionally need to be activated by signals from their microenvironment (i.e. interferon from lymphocytes, tumor cells, bacteria, foreign particles, or environmental toxins). Hence, macrophage activation is an essential cellular process underlying innate immunity (Adams, 1994; Barish, Downes, Alayick, Yu, Ocampo, Bookout, Mangelsdorf & Evans, 2005; Mosser & Edwards, 2008; Wynn et al., 2013).

Depending on the type of activating signals, monocyte-derived inflammatory macrophages can be polarized in functional phenotypes, and thus differ in terms of receptor expression, cytokine production, effector function, and chemokine repertoires (Mantonvani, Sica, Sozzani, Allavena, Vecchi & Locati, 2004; Zhang & Wang, 2014). Two well-established phenotypes are commonly referred to as *classically activated (M1) macrophages* and *alternatively activated (M2) macrophages* (Gordon & Taylor, 2005; Zhang & Wang, 2014). This M1 and M2 terminology reflects the T-helper 1 (Th1) / T-helper 2 (Th2) lymphocytes polarization scheme (Biswas et al., 2012; Hume & Freeman, 2014; see Figure 3) and differs in terms of polarizing signals, cytokine production, and function (Biswas et al., 2012).

2.1.2.1 Classically activated macrophages (M1 phenotype)

Classically activated macrophages are characterized by a high expression of the surface molecules major histocompatibility complex (MHC) class II and B7-2 (CD86; i.e. a costimulator for T-cell activation) and a low expression of mannose receptors (Mosser, 2003). Mannose receptor is a type I transmembrane glycoprotein that binds mannose and fucose residues on microbial walls and mediates phagocytosis (Abbas et al., 2007). Furthermore, classically activated macrophages show a change in their secretory profile pattern by secreting higher levels of proinflammatory cytokines such as interleukin (IL)-6, IL1ß, TNF-α, IL-12 and IL-23 as well as higher levels of chemokines such as CCL15, CCL20, CXCL9, and CXCL10 (Colin et al., 2014; Martinez & Gordon, 2014; Mosser & Edwards, 2008; Zhang & Wang, 2014). Notably, a higher expression of the different chemokines promotes the recruitment of Th1 cells, natural killer (NK) cells, and different leukocytes in order to drive cell inflammatory response forward (Colin et al., 2014). In addition to the higher proinflammatory cytokine and chemokine secretion, classically activated macrophages produce high amounts of highly oxidizing agents (i.e. reactive oxygen species (ROS) and nitrogen species (RNS)), which play a mediating role in microbicial activity, that is, the killing of microbes (Martinez, Sica, Mantovani & Locati, 2008).

Classically activated macrophages are formed in response to interferon-gamma (IFN-γ), alone or in combination with microbe or microbial product such as lipopolysaccharide (LPS), and other inflammatory cytokines like TNF-α (Colin et al., 2014; Mosser, 2003).

IFN-γ is known as the main cytokine associated with the conversion into classically activated macrophages (Mosser & Edwards, 2008) and is secreted by activated CD4+ Th1 cells, CD8+

T cytotoxic 1 cells, and NK cells (Martinez, Helming & Gordon, 2009). The binding of IFN-γ to its receptors (i.e. IFNGR-1 and IFNGR-2 subunits of the IFN-γ receptors) on the surface of macrophages leads to recruitment of Janus kinase (Jak) 1 und Jak2, in turn leading to the phosphorylation of signal transducers and activators of transcription 1 (STAT1). Phosphorylated STAT1 translocates to the cell nucleus and stimulates transcription of STAT1 target genes (Hu & Ivashkiv, 2009; Martinez & Gordon, 2014). In addition, IFN-γ enhances macrophage responsiveness to LPS by an up-regulation of the LPS receptor termed as Toll-like receptor 4 (TLR4; Hu & Ivashkiv, 2009; Schroder, Sweet & Hume, 2006). Notably, the secretion of IFN-γ during inflammation primes macrophages to synthesize proinflammatory cytokines (e.g. IL-6, IL1ß, and TNF-α) and to secrete increased amounts of ROS and RNS in order to enhance their killing ability (Colin et al., 2014; Mosser & Edwards, 2008; Zhang & Wang, 2014). For a sustained M1 population and thus a constant host defense, a sustained IFN-γ production by Th1 lymphocytes is needed (Mosser & Edwards, 2008; Mosser).

LPS is an important component of the cell membrane of Gram-negative bacteria (Hunter, Wang, Eubank, Baran, Nana-Sinkam & Marsh, 2009; Lu, Yeh & Ohashi, 2008) and is recognized by a cell surface receptor complex consisting of the TLR4 and its accessory protein MD-2 (Guha & Mackman, 2001). The activation of the TLR4-MD-2 complex induces the activation of several intracellular signaling pathways such as IkappaB kinase (IKK)-nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), mitogen-activated protein kinase (MAPK), and STAT1 (Guha & Mackman, 2001; Martinez & Gordon, 2014), which in turn lead to the activation of different transcription factors. The activation of these transcription factors coordinates the induction of numerous genes encoding inflammatory mediators (e.g. TNF-α; Guha & Mackman, 2001).

The proinflammatory cytokine TNF-α is secreted by macrophages themselves in response to stimulation with LPS (Takashiba, van Dyke, Amar, Murayama, Soskole & Shapira, 1999) and IFN-γ (Wynn et al., 2013). TNF-α also contributes to the formation of classically activated macrophages through the activation of intracellular signaling pathways (e.g. IKK-NF-κB, MAPK) indicated above (Takashiba et al., 1999). Furthermore, TNF-α has been shown to increase ROS production and thus enhance the microbicidal activity of classically activated macrophages supposedly by the activation of the NF-κB pathway (Gauss et al., 2007).

Notably, other cytokines (e.g. IL-10, transforming growth factor beta (TGFß)) inhibit the activation of classically activated macrophages (Abbas et al., 2007; Martinez & Gordon, 2014).

Functionally, classically activated macrophages are characterized by cellular immunity, proinflammatory cytokine production, and ROS/RNS-mediated microbicidal activity (Biswas et al., 2012; Cao et al, 2015; Dale et al., 2008; Gordon & Taylor, 2005). Thus, M1 macrophages are responsible for anti-microbial responses and tissue damage (Hunter et al., 2009). Notably, a chronic induction of M1 macrophage activation can cause chronic inflammation and tissue damage (Colin et al., 2014; Mosser, 2003; Mosser & Edwards, 2008).

2.1.2.2 Alternatively activated macrophages (M2 phenotype)

As this thesis focuses on M1 macrophages, this section only briefly describes M2 macrophages and their sub-groups.

In contrast to classically activated macrophages, alternatively activated macrophages or M2a macrophages are primarily formed in response to the cytokines IL-4 and IL-13 produced by Th2 cells, mast cells, eosinophils, basophils, NK cells, and even macrophages themselves (Colin et al., 2014; Gordon & Martinez, 2010; Martinez & Gordon, 2014). IL-4 and IL-13 down-regulate intracellular signaling pathways (e.g. NF-κB and STAT1) in order to inhibit the induction of inflammatory chemokines associated with the development of inflammation (Mantovani et al., 2004). M2a macrophages are characterized by the production of anti-inflammatory cytokines such as IL-10 (high production) and IL-12 (low production; Colin et al., 2014; Mantovani et al., 2004). In addition, they express high levels of scavenger, mannose, and galactose receptors (Biswas et al., 2012) and secrete pro-fibrotic factors (e.g. fibronetic), insulin-like growth factor (IGF), and TGFß (Colin et al., 2014; Zhang & Wang, 2014).
Depending on the activating stimuli, M2a macrophages are further classified into two sub-groups (Martinez et al., 2008): (1) M2b macrophages are induced by exposure to immune complexes in combination with LPS and IL-1ß, and (2) M2c macrophages are induced by IL-10, TGFß, or glucocorticoids. M2c macrophages display high expression levels of the Mer receptor tyrosine kinase (MerTK) to provide phagocytosis of apoptotic cells (Colin et al., 2014; Hunter et al., 2009).

Generally, alternatively activated macrophages are involved in the suppression of inflammation, tissue remodeling, wound healing, parasite clearance, tumor progression, and

immunoregulation (Biswas et al., 2012; Sica & Mantovani, 2012), and promote allergic responses (Hunter et al., 2009).

Figure 3 provides a simplified overview of the M1- and M2-polarized macrophages. However, M1 and M2 macrophages only represent two extremes of a linear scale (Biswas et al., 2012; Mosser & Edwards, 2008). This binary classification cannot represent the nuances that exist between macrophage populations (Wynn et al., 2013).

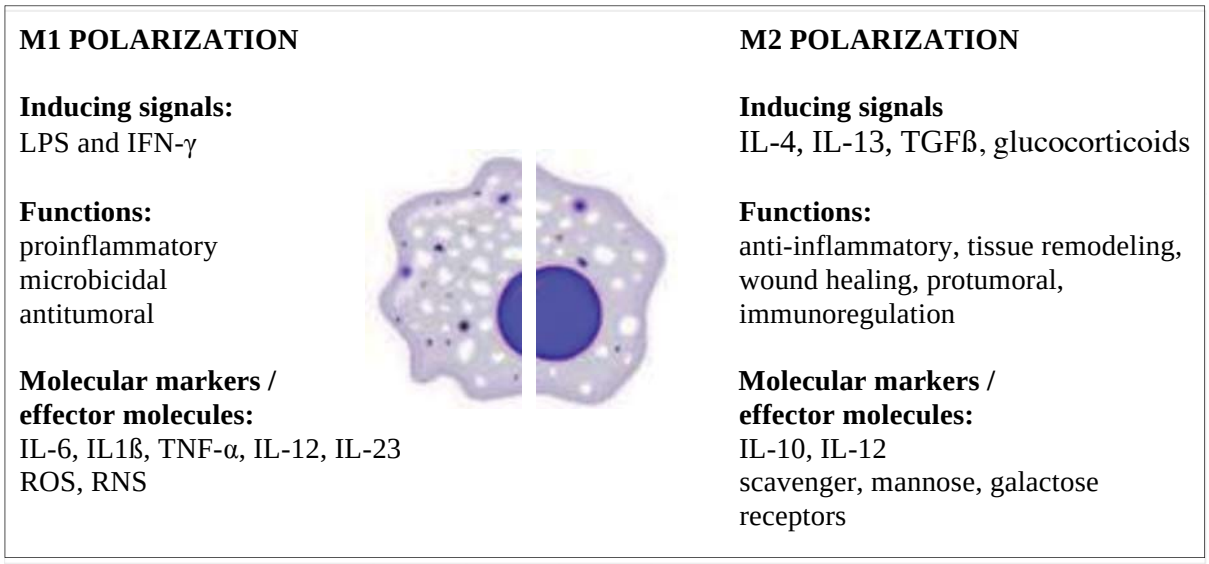

Figure 3: M1- and M2-polarized macrophages. Adapted from Biswas et al., (2012) and Mantovani, Sica & Locati (2007)

2.1.3 Microbicidal activity

Microbicidal activity is a key innate immune effector function of classically activated M1 macrophages (Colin et al., 2014; Mosser & Edwards, 2008; Zhang & Wang, 2014; see section 2.1.2.1). The killing of microbes, and thus microbicidal activity, is primary mediated by oxygen-dependent mechanisms. These mechanisms include the production of free radicals (Paulnock, 2000).

2.1.3.1 Free radicals

Free radicals are molecules containing one or more unpaired electrons, and include ROS and RNS (Halliwell, 2006; Hunter et al., 2009). Both ROS and RNS produced by M1 macrophages play a critical role in host defense (Nathan & Siloh, 2000).
ROS and RNS are collective terms and include both free radical and non-radical compounds (Halliwell, 2006). Notably, due to the unpaired electron, free radicals are highly reactive, unstable and short-lived, whereas non-radical derivatives are less reactive, more stable and long-lived (Paravicini & Touyz, 2008). Free oxygen radicals of ROS comprise, for example, superoxide (O_2^-), hydroxyl (OH), hydroperoxyl (HO_2), and carbonate (CO_3^-), whereas the non-radical oxygen derivatives include hydrogen peroxide (H_2O_2), hypobromous acid (HOBr), hypochlorous acid (HOCl), and ozone (O_3). Nitric oxide (NO), nitrogen dioxide (NO_2), and nitrate radical (NO_3) are known as free radical nitrogen compounds of RNS, whereas nitrous acid (HNO_2), dinitrogen tetroxide (N_2O_4), and dinitrogen trioxide (N_2O_3) are representatives of the non-radical nitrogen compounds (Halliwell, 2006).

Notably, two free radicals are able to join their unpaired electrons and form a covalent bond. Hence, ROS and RNS have common non-radicals such as peroxynitrite ($ONOO^-$) formed by the reaction of NO and O_2, peroxynitrate (O_2NOO^-) formed by the reaction of $ONOO^-$ and CO_2^-, and peroxynitrous acid (ONOOH) formed by the reaction of OH and NO_2 (Halliwell, 2006). Table 1 lists selected types of free radicals and non-radicals of ROS and RNS.

Table 1

Selected types of free radicals and non-radicals of ROS and RNS. Adapted from Halliwell (2006).

	Reactive oxygen species (ROS)	**Reactive nitrogen species (RNS)**
Free radicals	Superoxide (O_2^-) Hydroxyl (OH) Hydroperoxyl (HO_2) Carbonate (CO_3^-) Peroxyl (RO_2) Alkoxyl (RO) Carbon dioxide radical (CO_2^-) Singlet ($O_2{}^1\sum g^+$)	Nitric oxide (NO) Nitrogen dioxide (NO_2) Nitrate radical (NO_3)
Non-radicals	Hydrogen peroxide (H_2O_2) Hypobromous acid (HOBr) Hypochlorous acid (HOCl) Ozone (O_3) Organic peroxides (ROOH) Peroxomonocarbonate ($HOOCO_2$). Peroxynitrite ($ONOO^-$) Peroxynitrate (O_2NOO^-) Peroxynitrous acid (ONOOH)	Nitrous acid (HNO_2) Nitrosyl cation (NO^+) Nitroxyl anion (NO^-) Dinitrogen tetroxide (N_2O_4) Dinitrogen trioxide (N_2O_3) Nitronium cation (NO_2^+) Peroxynitrite ($ONOO^-$) Peroxynitrate (O_2NOO^-) Peroxynitrous acid (ONOOH)

Although both ROS and RNS are involved in oxygen-dependent mechanisms of microbicidal activity, the microbicidal effectiveness of M1 macrophages is largely due to their production

of ROS (de Oliveira-Junior et al., 2011; Halliwell, 2006). Therefore, in the following, the main source of ROS will be described.

2.1.4 Sources of reactive oxygen species

ROS are generated through a variety of sources, which are classified into intracellular and extracellular (Hunter et al., 2009). Intracellular sources comprise the mitochondria electron transport chain, cytochrome p450 (hemoproteins), lipoxygenase and cyclooxygenase pathway, xanthine oxidase complex, peroxisomes, and NADPH oxidase complex (Inoue, Sato, Nishikawa, Park, Kira, Imada & Utsumi, 2003). Extracellular sources include oxidative stress and chemical agents (Ueda, Masutani, Nakamura, Tanaka, Ueno & Yodoi, 2002).

The NADPH oxidase complex is a well-documented and major intracellular source of ROS (Iles & Forman, 2002). Notably, the classic NADPH oxidase was first described and characterized in phagocytes such as neutrophils, eosinophils, or macrophages and is known as *phagocytic* NADPH oxidase. However, studies indicated that similar NADPH oxidase systems are present in non-phagocytic cells such as fibroblasts, endothelial cells, or vascular smooth muscle cells (VSMCs). This NADPH oxidase type is called *non-phagocytic* or *vascular* NADPH oxidase (Quinn & Gauss, 2004).

2.1.4.1 Phagocytic NADPH oxidase

The NADPH oxidase is a multi-component enzyme complex (Babior, 2004) that plays an essential role in killing microbes by generating superoxide anions and other types of ROS (El-Benna, Dang, Gougerot-Pocidalo & Elbim, 2005). This multi-component enzyme is located in the plasma membrane and cytosol of unstimulated phagocytic cells (Vignais, 2002), and consists of two membrane-bound elements ($gp91^{phox}$ and $p22^{phox}$; **phox** for **ph**agocyte **ox**idase), three cytosolic components ($p67^{phox}$, $p47^{phox}$, and $p40^{phox}$) and a low-molecular-weight G protein (Rac1 in monocytes/macrophages or Rac2 in neutrophils; Babior, 2004; El-Benna et al., 2005).

Notably, in unstimulated cells, the NADPH oxidase is unassembled and inactive and its components are divided into plasma membrane and cytosolic locations (DeLeo, Allen, Apicella & Nauseef, 1999), i.e. $gp91^{phox}$ and $p22^{phox}$ are located in the plasma membrane,

whereas $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and Rac are located in the cytosol as a complex (Babior, 1999).

2.1.4.1.1 Phagocytic NADPH oxidase components

$gp91^{phox}$ and $p22^{phox}$

The two membrane-bound components $gp91^{phox}$ (also termed as Nox2) and $p22^{phox}$ compose the cytosolic core of the enzyme (Cachat, Deffert, Hugues & Krause, 2015). $gp91^{phox}$ is the most important element of NADPH oxidase (Babior, 2004) and is localized in intracellular and plasma membranes in close association with $p22^{phox}$ (Bedard & Krause, 2007). $gp91^{phox}$ contains the catalytic site and the NADPH-binding site, whereas $p22^{phox}$ is responsible for both $gp91^{phox}$ stability and the docking of $p47^{phox}$ (Cachat et al., 2015) in order to bring the cytosolic oxidase complex to the membrane to assemble the active oxidase (Babior, 2004). In contrast to $gp91^{phox}$, $p22^{phox}$ is not directly involved in the electron transfer (Nobuhisa Takeya, Oguras, Ueno, Kohda, Inagaki & Sumimoto, 2006). Both $gp91^{phox}$ and $p22^{phox}$ represent subunits of the heterodimer flavocytochrome b_{558} (Babior, 1999; Orient, Donko, Szabo, Leto & Geiszt, 2007). Flavocytochrome b_{558} comprise one flavin adenine dinucleotide (FAD) and two hemes and is the electron transfer chain of NADPH oxidase (Babior, 1999; El-Benna et al., 2005). Interestingly, $p22^{phox}$ was found in all major cellular components of the human vessel wall, i.e. endothelial cells, VSMCs, macrophages, and fibroblasts, indicating that $p22^{phox}$ is a common component of the *phagocytic* and *vascular* NADPH oxidase system (Azumi et al., 1999; Sorescu et al., 2002).

$p67^{phox}$

$p67^{phox}$ interacts with both Rac1/Rac2 and flavocytochrome b_{558} and regulates its catalytic activity (El-Benna et al., 2005). In addition, $p67^{phox}$ is suggested to be involved in the transfer of electrons directly from NADPH to oxygen to form superoxide (Babior, 2004).

$p47^{phox}$

$p47^{phox}$ binds to flavocytochrome b_{558} during activation and is responsible for transporting the cytosolic $p40^{phox}$- $p47^{phox}$- $p67^{phox}$ complex from the cytosol to the membrane during oxidase activation (Babior, 1999; El-Benna et al., 2005).

$p40^{phox}$

$p40^{phox}$ forms a part of the cytosolic oxidase subunit complex $p40^{phox}$- $p47^{phox}$- $p67^{phox}$. This component is not required for NADPH oxidase activation, and thus its function is still unclear (Babior, 2004; El-Benna et al., 2005; Quinn & Gauss, 2004). However, there are indications that $p40^{phox}$ plays a potential role in stabilization of the $p47^{phox}$- $p67^{phox}$ complex and in facilitating membrane recruitment of this complex during NADPH oxidase activation (Roos, van Bruggen & Meischl, 2003). Furthermore, $p40^{phox}$ is suggested to be both an activator and an inhibitor of NADPH oxidase (Babior, 1999; Groemping & Rittinger, 2005).

Rac

Rac belongs to the Rho-family of small GTPases, which is responsible for the regulation of a variety of signaling pathways (Groemping & Rittinger, 2005). As Rac interact with flavocytochrome b_{558} and $p67^{phox}$ and thus modulate the function of more than one NADPH oxidase protein (Quinn & Gauss, 2004), its presence is essential for optimal NADPH oxidase activation (El-Benna et al., 2005; Groemping & Rittinger, 2005).

2.1.4.1.2 Activation and assembly of the phagocytic NADPH oxidase

The role of NADPH oxidase is to catalyze the production of superoxide anions by transferring electrons from cytoplasmic NADPH to extracellular or intraphagolysosomal oxygen molecules (Babior, 2004; Cachat et al., 2015; Roos et al., 2003) according to the following reaction (Babior, 2004; de Oliveira-Junior et al., 2011; El-Benna et al., 2005; Roos et al., 2003):

$$NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^-$$

The superoxide anions formed in this reaction are further converted into hydrogen peroxide (H_2O_2) and other types of ROS (Babior, 2004; Cachat et al., 2015; de Oliveira-Junior et al., 2011). Thus, superoxide anions serve as a precursor to other, more reactive ROS (Babior, 1999; de Oliveira-Junior et al., 2011; Nobuhisa et al., 2006; Sheppard, Kelher, Moore, McLaughlin, Banerjee & Silliman, 2005).

In order to generate and provide agents to kill microbes, the unassembled and inactive NADPH oxidase needs to be activated (DeLeo et al., 1999). The NADPH oxidase activation is accompanied by two events: (1) protein phosphorylation and (2) translocation of cytosolic components to the plasma membrane (El-Benna et al., 2005).

Protein phosphorylation

Phosphorylation is one of the key events in NADPH activation (Babior, 1999; Groemping & Rittinger, 2005). The protein phosphorylation, and thus the activation of NADPH, depends on the binding of specific ligands to receptors expressed in the plasma membrane of phagocytic cells (i.e. macrophages; Vignais, 2002). Possible ligands are, for example, opsonized bacteria, opsonized zymosan, latex particles, complement fragment C5a, formylated peptides such as formylmethionine-leucyl-phenylalanine (FMLP), leukotriene B4 (LTB4), platelet-activating factor (PAF), diacylglycerol (DAG), calcium ionophores, protein kinase C (PKC) activators such as phorbol myristate acetate (PMA), and LPS (El-Benna et al., 2005). After the binding of a stimulus to a specific receptor, the receptor transmits information through the cytoplasmic membrane via GTP-binding proteins. These proteins in turn activate membrane enzymes such as phospholipase C, phospholipase A2, and phospholipase D, leading to the release of intracellular messengers (El-Benna et al., 2005), which activate kinases such as PKC, p21-activated kinase (PAC), p38 MAPK, PI-3K, and PA-activated protein kinase (Sheppard et al., 2005). Subsequently, the activated kinases catalyze the phosphorylation of the cytosolic NADPH oxidase subunits (Groemping & Rittinger, 2005). After the phosphorylation, the cytosolic components translocate to the plasma membrane (El-Benna et al., 2005).

Translocation of cytosolic components to the plasma membrane

Following the protein phosphorylation, the cytosolic oxidase subunits translocate en bloc to the membrane, where they associate with the membrane-bound heterodimer flavocytochrome b_{558} to assemble the active enzyme. In detail, during activation, $p47^{phox}$ is highly phosphorylated (Babior, 1999; El-Benna et al., 2005). The phosphorylation of $p47^{phox}$ leads to conformational changes that allow the interaction with flavocytochrome b_{558} and $p67^{phox}$ (de Oliveira-Junior et al., 2011; Fang, 2004). The resultant translocation of $p47^{phox}$ to the membrane assembles the other cytosolic components $p40^{phox}$, $p67^{phox}$, and Rac 1 or 2 (de Oliveira-Junior et al., 2011). Notably, $p47^{phox}$ by itself moves to the membrane to interact directly with flavocytochrome b_{558}, whereas $p67^{phox}$ and $p40^{phox}$ are recruited via their association with $p47^{phox}$. In contrast, Rac independently moves to the membrane and participates in the oxidase assembly (Nobuhisa et al., 2006). Taken toghether, the active NADPH oxidase enzymatic complex is formed by the translocation of the $p40^{phox}$- $p47^{phox}$-$p67^{phox}$ complex to the membrane-associated flavocytochrome b_{558} (see Figure 4). The active

NADPH oxidase complex transports electrons from cytoplasmic NADPH to extracellular or phagosomal oxygen to generate superoxide anions (Cachat et al., 2015; de Oliveira-Junior et al., 2011), which are of major interest for this thesis.

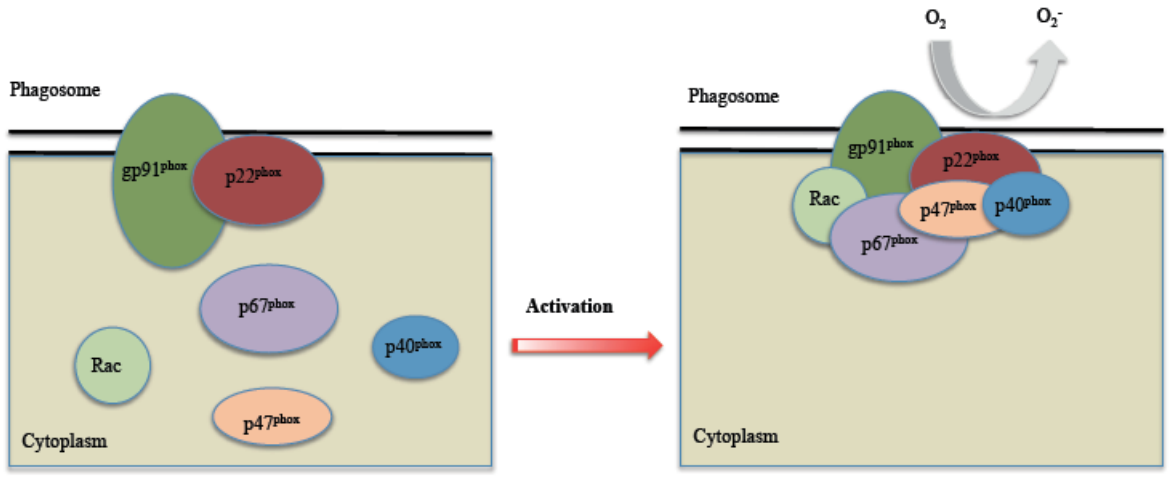

Figure 4: Phagocytic NADPH oxidase before and after activation. Adapted from de Oliveira-Junior et al. (2011).

2.1.4.2 Vascular NADPH oxidase

Although *vascular* NADPH oxidase is less important for this thesis, most of the studies reported in sections 2.2.5 and 2.3.4 are based on analyses of this type of NADPH oxidase. Therefore, *vascular* NADPH oxidase is described in the following.

Vascular NADPH oxidase-derived superoxide anions have been suggested to play an essential role in the development of hypertension, endothelial dysfunction, atherosclerosis, restenosis, and cardiac hypertrophy (Brandes & Kreuzer, 2005) and are produced by all vascular cell types, including fibroblasts, endothelial cells, and VSMCs (Paravicini & Touyz, 2008).

Vascular NADPH oxidases consist of the seven gp91^{phox} homologues Nox1 (**Nox** for **N**ADPH **ox**idase), Nox2, Nox3, Nox4, Nox5, Duox1, and Duox2 (Konior, Schramm, Czesnikiewicz-Guzik & Guzik, 2014), which are expressed in many tissues mediating different biological functions (Paravicini & Touyz, 2008). These vascular Nox isoforms have six transmembrane domains, which participate in electron transfer and thus lead to the reduction of molecular oxygen to superoxide anions (Konior et al., 2014). However, the catalytic subunit Nox2 of the *vascular* NADPH appears to be distinct from its *phagocytic* counterpart gp91^{phox} (van

Heerebeek, Meischl, Stooker, Meijer, Niessen & Roos, 2002). The main difference between *phagocytic* gp91^{phox} and *vascular* Nox isoforms is that ROS production by *phagocytic* gp91^{phox} occurs in the external face of the plasma membrane, releasing ROS into phagosomes or the extracellular medium, whereas ROS produced by *vascular* Nox isoforms are identified in the intracellular medium and are produced in smaller quantities (El-Benna et al., 2005).

2.1.4.2.1 Vascular NADPH oxidase components

Nox1

Nox 1 has been identified as the first homologue of gp91^{phox} and is expressed in endothelial cells, smooth muscle cells, and adventitial cells of the vasculature (Konior et al., 2014). Nox1-dependent ROS generation plays a critical role in cell signaling, cell growth, angiogenesis, and cell motility (Konior et al., 2014; Paravicini & Touyz, 2008). Nox1 primary produce superoxide anions (Konior et al., 2014) that are generated intracellularly (van Heerebeek et al., 2002). Furthermore, Nox1 needs p22^{phox}, p47^{phox} and p67^{phox} for its activation (Kleniewska, Piechota, Skibska & Goraca, 2012).

Nox2

Nox2 was initially termed gp91^{phox} and is the catalytic subunit of the *phagocytic* NADPH oxidase (Konior et al., 2014; Paravicini & Touyz, 2008; see section 2.1.4.1). It consists of the subunits gp91^{phox}, p22^{phox}, p47^{phox}, p67^{phox}, p40^{phox} and Rac 1 or 2 and is the most widely expressed *vascular* NADPH oxidase isoform. It is found in VSMCs, adventitial fibroblasts, endothelial cells, and perivascular adipocytes. Similar to Nox1, Nox2 primarily produce superoxide anions (Konior et al., 2014).

Nox3

Nox3 is found in inner ear and fetal tissue including kidney, liver, lung, and spleen (Kleniewska et al., 2012) and is involved in vestibular function (Paravicini & Touyz, 2008).

Nox4

Nox4, originally termed ‘Renox’ (renal oxidase), was initially detected in the kidney (Bedard & Krause, 2007). It is also found in VSMCs, fibroblasts, endothelial cells (Konior et al., 2014, Paravicini & Touyz, 2008), heart, pancreas, and osteoclasts (Kleniewska et al., 2012). Nox4 might be involved in stress signal transduction in the kidney and smooth muscle cells. To date, it is unclear which type of ROS is predominantly generated by Nox4 (Konior et al.,

2014), but there are some indications that Nox4 mainly generates hydrogen peroxide (Kleniewska et al., 2012).

Nox5

Nox5 is a calcium-dependent homologue (Brandes & Kreuzer, 2005; Kleniewska et al., 2012) and is mostly expressed in leukocytes, in the testis, in lymphatic tissue, endothelial cells, and VSMCs (Konior et al., 2014; Paravicini & Touyz, 2008). Nox5 appears to be the only *vascular* Nox isoform that can produce ROS, mainly superoxide anions and hydrogen peroxide, in the absence of other 'phox' or Rac subunits (Drummond & Sobey, 2014; Konior et al., 2014).

Duox1 and Duox2

Duox1 and Duox2 (for dual oxidases 1 and 2) are expressed in the thyroid gland and are involved in thyroid hormone biosynthesis (van Heerebeek et al., 2002; Paravicini & Touyz, 2008).

Notably, Nox1, Nox2, Nox4, and Nox 5 are commonly expressed in vascular cells, while Nox3, Duox1, and Duox2 have not been found or are expressed at low levels, meaning that their role is unclear (Konior et al., 2014).

2.1.4.2.2 Activation and assembly of the vascular NADPH oxidase

Due to the heterogeneity in vascular oxidase expression (Brandes & Kreuzer, 2005), *vascular* Nox isoforms have different mechanisms of activation (Konior et al., 2014) depending on vascular cell types (Brandes & Kreuzer, 2005; Konior et al., 2014).

Activation of *vascular* NADPH oxidases occurs in response to various agonists (Konior et al., 2014). Angiotensin II (Ang II) is the main agonist for oxidase activation in the vasculature (Brandes & Kreuzer, 2005). In the early phase of activation, Ang II-induced production of ROS is initially PKC-dependent (Konior et al., 2014). In detail, Ang II binds the G-protein-coupled AT1 receptor, which activates phospholipase C. The subsequent release of diacylglycerol activates PKC, which in turn phosphorylates $p47^{phox}$ (Brandes & Kreuzer, 2005). An enhanced and prolonged Ang II-induced production of ROS is dependent on the activation of Rac, Src kinase, and phosphatidylinositol 3-kinase (PI3-K; Brandes & Kreuzer, 2005; Konior et al., 2014).

2.1.5 Bacteria-killing mechanisms of reactive oxygen species

As described above, ROS, in particular superoxide anions, are critical components of the antimicrobial repertoire of phagocytic cells such as macrophages, but the mechanisms by which ROS damage bacteria in the phagosome are still not fully understood (Slauch, 2011).
ROS mainly have different target molecules including thiols, metal centers, and deoxyribonucleic acid (DNA; Fang, 2004). Based on studies in *Escherichia coli*, the main mechanism of ROS-dependent antibacterial activity is DNA damage (Fang, 2004; Slauch, 2011). DNA damage is dependent on the presence of iron. ROS can mobilize iron from iron-sulfur-containing dehydratases and potentiates its toxicity (Fang, 2004). However, pathogenic microorganisms have developed a range of strategies to resist the harmful action of ROS. These microbial-resistance strategies include evasion, suppression, enzymatic inactivation, scavenging, iron sequestration, stress responses, and repair mechanisms (Fang, 2004).

2.1.6 Clinical relevance of reactive oxygen species

Although the killing mechanisms of ROS, particularly superoxide anions, are not fully understood (Slauch, 2011), the clinical relevance of *phagocytic* NADPH oxidase-derived superoxide anion production in host defense is clearly demonstrated by a human genetic disorder called *chronic granulomatous disease* (CGD; El-Benna et al., 2005; Quinn & Gauss, 2004).
CGD is characterized by the failure of superoxide anion production by phagocytes (Babior, 1999; Babior, 2004; Cachat et al., 2015) due to defects in the genes that encode *phagocytic* NADPH oxidase subunits (Fang, 2004). Consequently, patients with CGD show an impaired destruction of microorganisms (El-Benna et al., 2005; Vignais, 2002) and have an increased susceptibility to life-threatening bacterial and fungal infections (Babior, 1999) including Aspergillus, Staphylococcus aureus, Burkholderia cepacia, Serratia marcescens, Nocardia (Cachat et al., 2015), and various Salmonella species (Roos et al., 2003). In addition, CGD presents most often with pneumonia, infectious dermatitis, osteomyelitis, and recurrent or severe abscess formation in the skin and organs of the reticuloendothelial system. The treatment of CGD includes prophylactic antibiotics, antifungals, and IFN-γ (de Oliveira et al., 2011). Although the treatment with IFN-γ may reduce the incidence of infection in CGD, the underlying mechanism is not understood (Fang, 2004).

Notably, NADPH oxidase-derived superoxide anions are not only clinically relevant in CGD, but also in hypertension, CAD, and atherosclerosis (Drummond & Sobey, 2014; see sections 2.2.5 and 2.3.4).

2.2 Essential hypertension

Arterial hypertension is a chronic medical condition characterized by a sustained elevation of BP in the arteries (Mancia et al., 2013; Nandhini, 2014). It is one of the primary risk factors for cardiovascular diseases (CVD), in particular CAD and its underlying process of atherosclerosis (Alexander, 1995; Hajjar et al., 2006; Mancia et al., 2013; Perk et al., 2012). Studies have indicated a strong positive association between BP and the risk of CVD and mortality, even in the normotensive range (Carretero & Oparil, 2000; Lewington, Clarke, Oizilbash, Peto & Collins 2002). Therefore, it is not surprising that in 2010 hypertension was identified as the single greatest risk factor globally, accounting for 9.4 million deaths and 7.0 % of global disability-adjusted life years (Lim et al., 2012). According to the World Health Organization's (WHO) World Health Day (2013), hypertension is responsible for at least 45% of deaths due to heart disease, and 51% of deaths due to stroke.

Analyses of worldwide data have shown that in the year 2000, there were 972 million people living with hypertension, and it is predicted that this number will increase to more than 1.56 billion in 2025 (Kearney, Whelton, Reynolds, Muntner, Whelton & He, 2005). According to the WHO Global status report on noncommunicable diseases (2014), the global prevalence of hypertension in adults (≥ 18 years) was 22%. Although the prevalence of uncontrolled hypertension decreased slightly between 1980 and 2008, the number of people with hypertension rose from 605 million in 1980 to 978 million in 2008 due to population growth and ageing (Danaei et al., 2011). The latest estimates regarding the prevalence of hypertension in the general European population range between 30 and 45%, with a steep increase with age (Mancia et al., 2013).

The high prevalence of hypertension should not be ignored because uncontrolled and untreated hypertension is a major cause of disability and premature death worldwide. Therefore, awareness, prevention, treatment, and control of hypertension are essential in order to reduce adverse health consequences (Chockalingam, Campbell & Fodor, 2006).

It should be noted that essential hypertension, also termed as primary hypertension or systemic hypertension, is defined by a rise in BP of unknown cause (Messerli et al., 2007) and includes over 90% of all hypertensive cases (Bolivar, 2013). Therefore, essential hypertension should be differentiated from secondary hypertension, which is defined as an increased BP of identifiable cause such as renal and vascular disease, obstructive sleep apnea, aldosteronism,

thyroid disease, or hypercortisolism. Secondary hypertension affects 5-10% of the general hypertensive population (Rimoldi, Scherrer & Messerli, 2013).

2.2.1 Definition and classification

BP is measured in millimeters of mercury (mm Hg) and is recorded as systolic (maximum) pressure over diastolic (minimum) pressure. The systolic blood pressure (SBP) measures the pressure in the arteries when the heart contracts, or beats, whereas the diastolic blood pressure (DBP) measures the pressure in the arteries between heartbeats when the heart muscle relaxes and refills with blood (WHO, 2013). The BP level depends on various parameters such as age (Mancia et al., 2013), gender (Sandberg & Ji, 2012) or lifestyle (e.g. alcohol consumption; Klatsky & Gunderson, 2008), and therefore shows a high inter- and intra-individual variability.

According to the European Society of Hypertension (ESH) – European Society of Cardiology (ESC) guidelines for the management of arterial hypertension, any numerical definition and classification of hypertension is arbitrary due to the continuous relationship between BP level and CVD risk (Mancia et al., 2003). In practice, however, cut-off BP values are needed to simplify the diagnostic approach and facilitate treatment decisions (Mancia et al., 2013). The definition and classification of hypertension in adults according to the ESH-ESC guidelines for the management of arterial hypertension (Mancia et al., 2013) is shown in Table 2. Hypertension is defined as values ≥140 mm Hg SBP and / or ≥ 90 DBP mm Hg. These recommendations are based on office BP measurements (i.e. BP measurements in a physisan's office or in a clinical setting). Isolated systolic hypertension should be graded as 1, 2, or 3 according to SBP values in the ranges indicated, provided diastolic values are < 90 (Mancia et al., 2013).

Table 2

Definitions and classifications of blood pressure levels (mm Hg). Adapted from Mancia et al. (2013).

Category	**Systolic**		**Diastolic**
1. Optimal	< 120	and	< 80
2. Normal	120-129	and / or	80-84
3. High normal	130 - 139	and / or	85 - 89
4. Grade 1 hypertension	140 - 159	and / or	90 - 99
5. Grade 2 hypertension	160 - 179	and / or	100 - 109
6. Grade 3 hypertension	≥ 180	and / or	≥ 110
7. Isolated systolic hypertension	≥ 140	and	< 90

Data from observational studies have shown that death from both ischemic heart disease and stroke increases progressively and linearly with elevation from normal BP (Lewington et al., 2002; MacMahon, Haijar & Rodgers, 2005). In addition, longitudinal data from the Framingham Heart Study indicated that CVD risk is increased 2.5-fold in women and 1.6-fold in men with high-normal as compared to normal BP levels (Vasan, Larson, Leip, Evans, O'Donnell, Kannel & Levy, 2001). Thus, the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure introduced a new classification, termed 'prehypertension', for BP levels ranging from SBP 120-139 mm Hg and / or DBP 80-89 mm Hg (Chobanian et al., 2003).

2.2.1.1 Total cardiovascular risk

Although the relationship between BP and cardiovascular risk is continuous, consistent, and independent of other risk factors, the majority of patients with hypertension show additional CVD risk factors, which potentiate their total cardiovascular risk (Chobanian et al., 2003; Mancia et al., 2013). Therefore, in 1994 the ESC, ESH, and European Atherosclerosis Society (EAS) developed recommendations for the prevention of CAD in clinical practice and emphasized that CAD prevention should be related to quantification of total cardiovascular risk. Moreover, therapeutic strategies should consider total cardiovascular risk in addition to BP values (Mancia et al., 2013). Although risk stratification of current guidelines for the

management of arterial hypertension is based on office BP measurement (Mancia et al., 2003; Mancia et al., 2007; Mancia et al., 2013), a recent meta-analysis showed that home BP measurement refines risk stratification compared to office BP measurements that are presumably associated with no or only moderately elevated risk (Asayama et al., 2014).

According to the ESH-ESC guidelines for the management or arterial hypertension, total cardiovascular risk is stratified in four categories (Mancia et al., 2013; Table 3): low, moderate, high, and very high risk. This classification is based on BP category, cardiovascular risk factors, asymptomatic organ damage, presence of diabetes, symptomatic CVD, or chronic kidney disease, and refers to the 10-year risk of CVD mortality (Manica et al., 2013).

Table 3

Stratification of total cardiovascular risk. Adapted from Mancia et al. (2013).

Other risk factors, asymptomatic organ damage or disease	*High normal* SBP 130-139 or DBP 85-89 mmHg	*Grade 1 hypertension* SBP 140-159 or DBP 90-99 mmHg	*Grade 2 hypertension* SBP 160-179 or DBP 100-109 mmHg	*Grade 3 hypertension* SBP ≥180 or DBP ≥ 110 mmHg
No other risk factors		Low risk	Moderate risk	High risk
1-2 risk factors	Low risk	Moderate risk	Moderate to high risk	High risk
≥ 3 risk factors	Low to moderate risk	Moderate to high risk	High risk	High risk
Organ damage, chronic kidney disease stage 3 or diabetes	Moderate to high risk	High risk	High risk	High to very high risk
Symptomatic cardiovascular disease, chronic kidney disease stage ≥ 4 or diabetes with organ damage / risk factors	Very high risk	Very high risk	Very high risk	Very high risk

2.2.2 Diagnostic evaluation

The aims of an initial evaluation are to (I) diagnose hypertension, (II) identify potential causes of secondary hypertension, and (III) assess the overall CVD risk by searching for other risk factors, target organ damage, and concomitant clinical conditions (Mancia et al., 2013). Therefore, the diagnostic procedure includes the following medical examinations: (1) repeated BP measurements, (2) assessment of medical history including family history, (3)

physical examination, and (4) laboratory investigations (Mancia et al., 2003; Mancia et al., 2013)

2.2.2.1 Blood pressure measurement

BP measurement is one of the most commonly used medical tests worldwide (Kaczorowski, Dawes & Gfeller, 2012). BP can be measured in various ways: (1) by the physician or the nurse in the physician's office or in the clinic (office BP), (2) by the patient at home (home BP), and (3) automatically over a 24-h period (ambulatory BP; Mancia et al., 2013). Regardless of which measurement device is used, BP will always be influenced by many factors (e.g. exercise, meals, tobacco, alcohol; O'Brien et al., 2003) and will vary both within and between days (Manica et al., 2003). If these influences are ignored or unrecognized, erroneous diagnosis and inappropriate management may result (O'Brien et al., 2003). Therefore, accurate measurement is the basis of optimal diagnosis and treatment of hypertension (Kaczorowski et al., 2012).

For an accurate measurement of BP in the physician's office and at home, the following recommendations are used: Patients should be seated quietly for at least five minutes with their feet on the floor and arm supported at heart level. Caffeine, exercise, and smoking should be avoided for at least thirty minutes prior to measurement. Furthermore, an appropriately sized cuff should be used, and the average of two measurements at 1-minute intervals should be recorded (Chobanian et al., 2003). Multiple BP measurements, taken on several separate occasions, are needed for the diagnosis of hypertension (Mancia et al., 2003; Perk et al., 2012).

Office blood pressure measurement

Auscultatory or oscillometric semiautomatic sphygmomanometers represent the gold standard for measuring BP in clinical practice (Mancia et al., 2013; Perk et al., 2012). Whereas the auscultatory method, also known as the Riva-Rocci-Korotkoff method, is based on the detection of characteristic sounds (i.e. Korotkoff sounds) heard through a stethoscope applied over the brachial artery, the oscillometric method is based on air volume variations in the cuff detected during deflation (Bonnafoux, 1996). Both devices should be validated according to standardized protocols and checked periodically through calibration in a technical laboratory. If technically feasible, automated recording of multiple BP readings in the office might be

considered in order to improve reproducibility (Mancia et al., 2013). Devices measuring BP on the fingers or the wrist are inaccurate and should be avoided (Perk et al., 2012).

For a correct and accurate BP measurement in a physician's office or in a clinic, patients should sit for 3 to 5 minutes before BP measurement starts. It is considered necessary to take at least two BP measurements with 1-2-minute intervals in the sitting position. Additional BP measurements are needed if the first two measurements are different (Manica et al., 2013). Notably, measurement 1 minute and 3 minutes after assuming a standing position is recommended, for example, in elderly persons or diabetic patients (Mancia et al., 2013).

Home blood pressure measurement

Although office BP measurement shows higher BP values than home BP measurement (Verberk, Kroon, Kessels & de Leeuw, 2005), self-measurement of BP at home is of clinical value (Mancia et al., 2007). The major advantage of home BP measurements is that it provide a large number of readings over a period of several days (Mancia et al., 2013). In addition, home BP measurement correlates better with target organ damage and cardiovascular mortality than office BP measurement; it identifies normotension with almost absolute certainty, it enables prediction of sustained hypertension in patients with borderline hypertension, and it is an appropriate tool for assessing drug efficacy (Verberk et al., 2005). Moreover, a recent meta-analysis of prospective studies indicates that home BP measurement is a better predictor of cardiovascular mortality and cardiovascular events than office BP measurement (Ward, Takahashi, Stevens & Heneghan, 2012). Therefore, home BP measurement may represent a more reliable assessment than office BP measurement (Mancia et al., 2013).

To ensure a correct and accurate measurement of BP at home, it is essential to use a validated semi-automatic device and adequately explain the procedure to the patient, with verbal and written instructions (Mancia et al., 2013). Although a recent study has shown that even a single home BP measurement in the morning is a potent predictor of cardiovascular events, a mean of seven home measurements performed during seven days may be needed to reliably diagnose hypertension (Niiranen, Asayama, Thijs, Johansson, Hara, Hozawa, Tsuij, Ohkubo, Jula, Imai, Staessen & IDHOCO Investigators, 2015). It should be noted that home BP measurement is not indicated if it causes anxiety to the patient or if it induces self-modification of the treatment regimen (Mancia et al., 2007).

24-hour ambulatory blood pressure monitoring

24-hour ambulatory BP monitoring provides information about BP during daily activities and at night during sleep and is of prognostic significance (Mancia et al., 2013). Meta-analyses of published cohort studies in the general population and in hypertensive patients reported that 24-hour BP is a strong predictor of cardiovascular events, providing prognostic information independent of office BP (Boggia, et al., 2007; Conen & Bamberg, 2008; Hansen, Li, Boggia, Thijs, Richart & Staessen, 2011). Moreover, in hypertensive patients without major cardiovascular disease, both daytime and nighttime ambulatory BP significantly predict all-cause and cardiovascular mortality, CAD, and stroke, whereas only nighttime ambulatory BP predicts non-cardiovascular mortality. Furthermore, the night-day BP ratio for death, CVD, CAD, and stroke only persists for all-cause mortality after adjustment for 24-hour ambulatory BP (Fagard, Celis, Thijs, Staessen, Clement, De Buyzere & De Bacquer, 2008). However, night-day BP ratio in the general population and in hypertensive patients added little prognostic value over and beyond the 24-hour BP level (Hansen et al., 2011).

According to the ESH-ESC practice guidelines for the management of arterial hypertension, 24-hour ambulatory BP monitoring is indicated under several conditions: (1) considerable variability of office BP, (2) high office BP in subjects at low total cardiovascular risk, (3) large variability between home and office readings, (4) elevated office BP in pregnant woman, (5) resistance to drug treatment, and (6) suspected hypotensive episodes, sleep apnea, or preeclampsia (Manica et al., 2007). For an accurate ambulatory BP measurement, patients are instructed to engage in normal activities but to refrain from strenuous exercise. Furthermore, at the time of cuff inflation, patients should stop moving and talking and keep the arm still with the cuff at heart level. To increase the accuracy of 24-hour ambulatory BP measurements, it is recommended that the readings are made at the same frequency during the day and night (Mancia et al., 2013).

Table 4 shows the cut-off values for the classification of hypertension depending on the method of BP measurement.

Table 4

Blood pressure cut-off values (mm Hg) for definition of hypertension depending on the method of blood pressure measurement. Adapted from Mancia et al. (2013).

Category	**Systolic**		**Diastolic**
Normotension	< 120	and	< 80
Hypertension			
Office blood pressure	≥ 140	and / or	≥ 90
Home blood pressure	≥ 135	and / or	≥ 85
24-hour ambulatory blood pressure			
Daytime blood pressure	≥ 135	and / or	≥ 85
Nighttime blood pressure	≥ 120	and / or	≥ 70
24-hour	≥ 130	and / or	≥ 80

Although the diagnosis of hypertension is still based on office BP (Perk et al., 2012; Staessen, Wang, Bianchi & Birkenhäger, 2003), home BP measurement or 24-hour ambulatory BP measurement are indicated to exclude white-coat or masked hypertension (Mancia et al., 2013).

2.2.2.1.1 White-coat hypertension

White-coat hypertension is characterized by a higher office BP (> 140/90 mm Hg), with normal home or ambulatory BP values (< 135/85 mmHg) and the absence of end organ damage (Perk et al., 2013). The most suitable methods for identifying white-coat hypertension are 24-hour ambulatory (Staessen et al., 2003; Verberk et al., 2005) and home BP measurement (Stergiou, Siontis & Ioannidis, 2010).

The incidence of cardiovascular events does not significantly differ between white-coat hypertension and normotension (Fagard & Cornelissen, 2007). However, the majority of cross-sectional studies reported increased target organ damage in patients with white-coat hypertension (Franklin, Thijs, Hansen, O'Brien & Staessen, 2013). Furthermore, white-coat hypertension is associated with an increase in left ventricular mass and an increased prevalence of left ventricular hypertrophy (LVH, i.e. the major manifestation of hypertensive heart disease; Muscholl, Hense, Bröckel, Döring, Riegger & Schunkert, 1998). In addition,

carotid intima-media thickness (IMT) is greater and grows faster in white-coat hypertensive patients compared to normotensives (Puato, Palatini, Zanardo, Dorigatti, Tirrito, Rattazzi, Pauletto, 2008).

2.2.2.1.2 Masked hypertension

Masked hypertension is defined as a normal BP in the office (< 140/90 mm Hg), but a higher home or ambulatory BP (> 135/85 mm Hg; Pickering, Eguchi & Kario, 2007). Although 24-hour ambulatory BP measurement is recognized as the gold standard for identifying masked hypertension, home BP measurement identifies it in fairly similar ways (Bobrie, Clerson, Ménard, Postel-Vinay, Chatellier & Plouin, 2008) and might be recommended when 24-hour ambulatory BP measurement is not available or is unacceptable for patients due to the discomfort it may cause (Yano & Bakris, 2013).

Masked hypertension is a lesser-known but no less frequent clinical condition with a more serious prognosis than white-coat hypertension (Messerli et al., 2007). A meta-analysis of prospective studies indicated that the incidence of cardiovascular events is twice as high in masked hypertension compared to normotension (Fagard & Cornelissen, 2007). In addition, masked hypertension is associated with greater left ventricular mass and carotid wall thickness (Liu, Roman, Pini, Schwartz, Pickering & Devereux, 1999; Hernàndes del-Rey, Armario, Martin-Baranera, Sànchez, Almedros, Coca & Pardell, 2003), and therefore a higher prevalence of LVH (Sega et al., 2001).

2.2.2.2 Medical and family history

According to the ESH-ESC guidelines for the management of arterial hypertension, the medical history should include duration and previous level of high BP, including measurements at home, indications of secondary hypertension (e.g. history of renal disease, drug or substance intake, or repetitive episodes of sweating, headache, anxiety), lifestyle / risk factors (e.g. smoking, alcohol intake, physical activity, obesity), history and symptoms of organ damage and CVD, and hypertension management (e.g. current and past antihypertensive medication; Mancia et al., 2003; Mancia et al., 2013). Within the medical history, a family history with particular attention to hypertension, diabetes, dyslipidemia, premature CVD, stroke, or renal disease should be obtained (Mancia et al., 2003). Premature

hypertension and / or premature CVD are important indicators of genetic predisposition to hypertension and CVD and may indicate genetic tests (Mancia et al., 2013).

2.2.2.3 Physical examination

The physical examination aims to verify a diagnosis of hypertension with repeated BP measurements, establish current BP, and screen for potential secondary causes of hypertension, organ damage, and obesity. Therefore, height, weight, and waist circumference should be taken, body mass index (BMI) should be calculated, and all patients should undergo auscultation of the carotid artery as well as heart, and renal arteries. Furthermore, heart rate (HR) should be measured, because an increased HR indicates an increased risk of heart disease (Mancia et al., 2013).

Both medical / family history and physical examination should help to identify known and remediable causes of high BP, establish the presence or absence of target organ damage and CVD, and detect other CVD or comorbid conditions that might affect prognosis and treatment of hypertension (Carretero & Oparil, 2000).

2.2.2.4 Laboratory investigations

Laboratory investigations are needed to provide evidence for the presence of additional risk factors, testing for secondary hypertension, and assessing the absence or presence of organ damage. Investigations should progress from the simplest (e.g. blood chemistry) to the more complicated (e.g. tests of cerebral, cardiac, and renal functions; Mancia et al., 2013). Routine laboratory analyses to exclude secondary hypertension are recommended before initiating therapy (Chobanian et al., 2003), and include a 12-lead electrocardiogram, urine analysis, fasting plasma glucose, hemoglobin and / or hematocrit, serum potassium and sodium, serum uric acid, serum creatinine, and a lipoprotein profile after 9- to 12-hour fasting including serum total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and fasting serum triglycerides (Chobanian et al., 2003; Mancia et al., 2013). Additional tests are based on medical and family history, physical examination, and routine laboratory tests (Mancia et al., 2013). All laboratory investigations are indicated for the diagnosis of secondary hypertension and / or comorbid conditions (Carretero & Oparil, 2000).

2.2.3 Risk factors of essential hypertension

Although the origin of high BP is unknown, essential hypertension is understood as a multifactorial disease arising from the combined action of many genetic, environmental, and behavioral factors (Bolivar, 2013). A number of risk factors underlying hypertension have been identified, including non-modifiable risk factors such as age, gender, genetics, and ethnicity, as well as modifiable risk factors such as overweight / obesity, alcohol consumption, smoking, or job strain (Carretero & Oparil, 2000; Slama, Susic & Frohlich, 2002).

2.2.3.1 Non-modifiable risk factors

2.2.3.1.1 Age

Many cross-sectional and longitudinal studies have shown a continuous increase of SBP and DBP with ageing (Hajjar et al., 2006), and thus an increased incidence and prevalence of hypertension in the elderly (Slama et al., 2002). However, data from the Framingham Heart Study reported changing patterns of BP with ageing. In detail, DBP rises until approximately age 50 and slowly decreases from the age of 60 to at least 84 years of age, whereas SBP rises continuously throughout life (Chobanian et al., 2003; Pinto, 2007). Therefore, SBP is a more potent cardiovascular risk factor after the age of 50 (Chobanian et al., 2003). In addition, isolated systolic hypertension is more prevalent in those individuals aged 50 or over (Pinto, 2007). Clinical studies reported that control of isolated systolic hypertension reduces total mortality, cardiovascular mortality, stroke, and heart failure events (Chobanian et al., 2003). Notably, the age-specific increase in BP is shown in both sexes and all ethnicities (Wright, Hughes, Ostchega, Yoon & Nwankwo, 2011).

2.2.3.1.2 Gender

Cross-sectional studies reported gender-associated differences in BP, with men having higher BP than age-matched pre-menopausal women (Hajjar et al., 2006; Pechère-Bertschi & Burnier, 2004; Reckelhoff, 2001). After menopause, BP increases in women to levels that are even higher than those in men (Reckelhoff, 2001). Consequently, after the age of 60, women show a higher BP and therefore a higher prevalence of hypertension (Hajjar et al., 2006; Sandberg & Ji, 2012).

The gender-related increase in BP is also reflected in the incidence of hypertension: At the age of 30-39 years, the 2-year incidence rates were higher in men (3.3%) than in women (1.5%). At the age of 70-79 years, however, the 2-year incidence rates were higher in woman (8.6%) than in men (6.2%; Hajjar et al., 2006). The lifetime risk for developing hypertension for middle-aged and elderly men and women is 90% (Vasan, Beiser, Sehadri, Larson, Kannel, D'Agostino & Levy, 2002).

Although the underlying mechanisms responsible for the gender differences in BP are not fully understood (Pechère-Bertschi & Burnier, 2004), several determinants such as the presence or absence of testosterone or estrogens are suggested (Maranon & Reckelhoff, 2013).

2.2.3.1.3 Genetics

Essential hypertension is a highly heterogeneous disease with a multifactorial etiology (Mancia et al., 2013), in which both environmental and genetic factors play an essential role (Kamble, Mohod & Kumar, 2011). Almost 30% to 60% of the BP variation in the population is explained by genetic factors, with environmental exposure explaining the remaining part (Melander, 2001).

The influence of genes on BP has been shown by family studies demonstrating associations of BP among siblings and between parents and children (Carretero & Oparil 2000). The estimated heritability of BP based on twin studies is 50% to 70% (Melander, 2001). The concordance of BP is greater in monozygotic than dizygotic twins (Oparil, Zaman & Calhoun, 2003). Furthermore, population studies demonstrate greater similarity in BP within families than between families (Lifton, Gharavi & Geller, 2001; Oparil et al., 2003). This observation is not merely attributable to a shared environment, as adoption studies reported greater concordance of BP among biological siblings than adoptive siblings living in the same household (Lifton et al., 2001; Oparil et al., 2003). The estimated heritability in adoption studies is 34% to 44% for SBP and 30% to 34% for DBP (Melander, 2001).

In addition to twin or adoption studies, a family history of hypertension also provides information about heritability (Mancia et al., 2013; Melander, 2001). Individuals with two or more hypertensive first-degree relatives have been shown to be almost four times more likely to develop hypertension by the age of 40 (Melander, 2001).

2.2.3.1.4 Ethnicity

Most studies on ethnic differences in BP were conducted in the United States (Jones & Hall, 2006). Generally, African Americans have higher levels of SBP and DBP, both at night and during the day, compared to European Americans, with greater ethnic differences at night than during the day (Profant & Dimsdale, 1999; Wang, Treiber, Harshfield, Hanevold & Snieder, 2006). Moreover, a blunting of nocturnal BP declines was observed in African Americans (Wang et al., 2006). This blunting of nighttime decline in BP begins by the age of 10 years, is exacerbated with ageing (Wang et al., 2006), and is further associated with higher rates of vascular diseases (Jones & Hall, 2006). Furthermore, the lack of nocturnal decline in BP is not seen in persons of African descent outside the United States. Therefore, the blunting of nocturnal decline in BP does not seem to be purely genetic in origin, but rather appears to be the result of a gene-environment interaction (Jones & Hall, 2006). Possible environmental factors contributing to higher BP in African Americans are obesity and low socioeconomic status (Hajjar et al., 2006).

The prevalence of hypertension also varies across ethnic groups, reflecting the ethnic differences in BP. In the Multi-Ethnic Study of Atherosclerosis conducted in the United States, the prevalence of hypertension in African Americans was higher (60%) than in Whites (38%). The prevalence in Hispanic (42%) and Chinese (39%) individuals, however, did not significantly differ from that in Whites (Kramer et al., 2004). A recent cross-sectional study showed that Hispanic ethnicity (18.9%) compared to non-Hispanic Whites (27.7%) or non-Hispanic Blacks (35.5%) was associated with the lowest prevalence of hypertension. These associations persist even after controlling for possible confounding variables such as sex, age, BMI, education level, lack of physical activity, and alcohol consumption (Holmes, Hossain, Ward & Opara, 2013).

2.2.3.2 Modifiable risk factors

2.2.3.2.1 Overweight / obesity

Overweight is defined by a BMI between 25 and 29.9, while obesity is defined by a BMI of 30 or higher. Overweight / obesity is known as one of the major risk factors for hypertension (Harsha & Bray, 2008).

A positive relationship between overweight / obesity, BP, and the risk for hypertension was first reported in the early 1920s (Symonds, 1923), and was confirmed by epidemiological

studies in the following decades (Harsha & Bray, 2008). The Framingham Study reported that hypertension is approximately twice as prevalent in obese compared to non-obese individuals (Hubert, Feinleib, McNamara & Castelli, 1983; Harsha & Bray, 2008). Furthermore, overweight and obesity were highly related to the risk of hypertension in both men and women. The age-adjusted risk of hypertension in persons with a BMI of 30 or greater was 2.23 in men and 2.63 in women (Wilson, D'Agostino, Sullivan, Parise & Kannel, 2002).
The underlying mechanism by which overweight / obesity raises BP is not fully understood (Carretero & Oparil, 2000). Numerous potential biological mechanisms have been proposed, such as insulin resistance, enhanced sodium retention, or increased activation of sympathetic nervous system (SNS; Harsha & Bray, 2008).

2.2.3.2.2 Alcohol consumption

Alcohol consumption is a further important risk factor for the development of hypertension (Fuchs, Chambless, Whelton, Nieto & Heiss, 2001) and is the primary reversible cause of more than 5% of all hypertensive cases (Klatsky, 2003; Klatsky & Gunderson, 2008).
The positive relationship between alcohol consumption and BP elevation is well documented (Fuchs et al., 2001; Klatsky & Gunderson, 2008), with an increase in BP of 1 mm Hg for every 10 g of alcohol (Puddey & Beilin, 2006). However, the positive association between alcohol consumption and BP is only found in heavy drinkers (≥ 3 drinks / day), independent of alcoholic beverage type (Klatsky, 2003; Puddey & Beilin, 2006), race, and gender (Klatsky & Gunderson, 2008). Therefore, an excessive and chronic alcohol consumption of three or more drinks per day is related to an increased incidence (Fuchs et al., 2001) and prevalence (Klatsky & Gunderson, 2008; Puddey & Beilin, 2006) of hypertension. Furthermore, the association between high levels of alcohol consumption and increased risk of hypertension might depend on alcohol drinking patterns (Strangers et al., 2004): Individuals who mostly drink outside mealtimes showed a significant increase in the risk of hypertension compared to lifetime abstainers or those who mostly drink during mealtimes. This effect was independent of the amount of alcohol consumed and was present even in individuals with light to moderate alcohol intake (Strangers et al., 2004).

2.2.3.2.3 Cigarette smoking

Although cigarette smoking is one of the most important risk factors for CVD, its relationship with hypertension is inconsistent (Bowman, Gaziano, Buring & Sesso, 2007; Leone, 2011). Studies indicated that cigarette smoking is independently associated with an increased risk of hypertension in men who smoke 20 or more cigarettes per day (Niskanen et al., 2004) and a moderately increased risk in women who smoke at least 15 cigarettes per day (Bowman et al., 2007). On the contrary, other studies have shown lower BP in smokers compared to non-smokers (Green, Jucha & Luz, 1986; Mikkelsen et al., 1997). Furthermore, the period of smoking cessation is associated with both a progressive increase in BP and a higher incidence of hypertension (Lee, Ha, Kim & Jacobs, 2001).

Even though the association between cigarette smoking and BP and is not clarified (Bowman et al., 2007; Leone, 2011), cigarette smoking potentiates the enhanced cardiovascular risk in hypertensive individuals (Tanus-Santos, Toledo, Cittadino, Sabha, Rocha & Moreno, 2001). Therefore, cardiovascular mortality is highest in smokers with hypertension (Hozawa et al., 2007; Thomas, Rudnichi, Bacri, Bean, Guize & Benetos, 2001).

2.2.3.2.4 Job strain

Job strain is the most studied work-related stressor (Landsbergis, Dobson, Koutsouras & Schnall, 2013) and can result from a combination of high workload and few decision-making opportunities in the workplace (Babu et al., 2013). Although job strain is reported to be a risk factor for BP elevation, the findings regarding this relationship are inconsistent (Landsbergis et al., 2013). Cross-sectional, population-based, and prospective studies reported a significantly independent and positive association between job strain and BP (Cesena, Sega, Ferrario, Chiodini, Corrao & Mancia, 2003; Clays, Leynen, De Bacquer, Kornitzer, Kittel, Karasek & De Backer, 2007; Guimont, Brisson, Dagenais, Milot, Vézina, Masse, Moisan, Laflamme & Blanchette, 2006). This association was found not only with BP measurements at work but also with BP measurements at home and during sleep (Clays et al., 2007). By contrast, numerous studies indicated no association between job strain and BP elevation (Mann, 2006; Nyberg et al., 2013; Rosenthal & Alter, 2012; Sparrenberger et al., 2009; Spruill, 2010). This discrepancy might be due to methodological differences in BP measurement: In studies using office BP measurement, the evidence concerning job strain and BP elevation is inconsistent, whereas in studies using ambulatory BP measurement, the

association is much more consistent (Clays et al., 2007; Landsbergis et al., 2013). However, a recent meta-analysis confirmed that job strain is an independent risk factor for BP elevation during work, home, and sleep hours (Landsbergis et al., 2013). Furthermore, job strain was found to be significantly associated with hypertension in both case-control and cohort studies (Babu et al., 2013).

It should be noted that many of these modifiable risk factors are additive, such as overweight / obesity and alcohol consumption (Carretero & Oparil, 2000).

2.2.4 Treatment strategies

The primary goal of treatment in all hypertensive patients is to achieve the maximum reduction in the long-term total risk of cardiovascular morbidity and mortality (Mancia et al., 2003; Mancia et al., 2007). This requires treatment of the raised BP (at least below 140 mm Hg of SBP and 90 mm Hg of DBP) as well as of all associated reversible risk factors (Mancia et al., 2007). Treatment strategies include lifestyle modification and pharmacological therapy (Chobanian et al., 2003; Mancia et al., 2013; Perk et al., 2012).

2.2.4.1 Lifestyle modifications

Appropriate lifestyle modifications are the cornerstone for the management and prevention of hypertension (Mancia et al., 2013). Lifestyle measures should be assessed in all patients, including those with high normal BP and those who require drug treatment (Mancia et al., 2003). Notably, often, lifestyle interventions alone may be sufficient for patients with mildly elevated BP (Perk et al., 2012).

The following lifestyle modifications are known to lower BP and / or cardiovascular risk, and are therefore recommended in all patients (Mancia et al., 2013): (1) reduction of alcohol consumption, (2) weight reduction and its maintenance in overweight and obese hypertensive patients, (3) high consumption of vegetables and fruits and low-fat and other types of diet, (4) regular physical exercise, and (5) smoking cessation. A meta-analysis of 15 randomized controlled trials regarding the effects of alcohol reduction of more than one week on BP reported that an alcohol reduction of 67% was associated with a significant reduction in SBP (3.31 mm Hg) and DBP (2.04 mm Hg) independent of the intervention duration (Xin, He, Frontini, Ogden, Motsamai & Whelton, 2001). Furthermore, a meta-analysis of 25

randomized controlled trials concerning the influence of weight reduction on BP showed that a weight loss of 5.1 kilograms (kg) reduced SBP by 4.44 mm Hg and DBP by 3.57 mm Hg, (Neter, STam, Kok, Grobbee & Geleijnse, 2003). Moreover, BP also benefited from adoption of the Dietary Approaches to Stop Hypertension (DASH) eating plan (i.e. low-fat dairy products, fruits, and vegetables) and regular physical exercise (Chobanian et al., 2003; Mancia et al., 2013). The ENCORE study showed that the combination of the DASH diet with regular physical exercise and weight loss resulted in a greater BP reduction compared to DASH diet alone in overweight or obese individuals with high normal BP (Blumenthal et al., 2010). Additionally, to reduce the overall cardiovascular risk, hypertensive individuals should be strongly advised to quit smoking (Chobanian et al., 2003; Mancia et al., 2013).

2.2.4.2 Pharmacological therapy

The main benefits of antihypertensive treatment are due to the lowering of BP per se, and are largely independent of the drugs employed (Mancia et al., 2013; Perk et al., 2012). According to the current ESH-ESC guidelines for the management of arterial hypertension, the following antihypertensive drugs are suitable for the initiation and maintenance of antihypertensive treatment, either as monotherapy or in combination (Mancia et al., 2013): (1) diuretics, (2) beta-blockers, (3) calcium antagonists, (4) angiotensin-converting enzyme (ACE) inhibitors, and (5) angiotensin receptor blockers. More than two thirds of hypertensives cannot be normalized by one drug (monotherapy) and will therefore require two or more antihypertensive drugs selected from different drug classes (Chobanian et al., 2003).

Table 5 summarizes the recommendations on initiation of lifestyle modifications and antihypertensive drug treatment. Notably, despite the therapeutic advances in terms of lowering BP, hypertension continues to be a major public health problem with increasing prevalence worldwide (Chobanian, 2009).

Table 5

Initiation of lifestyle modifications and antihypertensive drug treatment. Adapted from Mancia et al. (2013).

Other risk factors, asymptomatic organ damage or disease	*High normal* SBP 130-139 or DBP 85-89 mmHg	*Grade 1 hypertension* SBP 140-159 or DBP 90-99 mmHg	*Grade 2 hypertension* SBP 160-179 or DBP 100-109 mmHg	*Grade 3 hypertension* SBP ≥180 or DBP ≥ 110 mmHg
No other risk factors	No BP intervention	1. Lifestyle modifications for several months 2. Then add BP drugs targeting < 140/90	1. Lifestyle modifications for several weeks 2. Then add BP drugs targeting < 140/90	1. Lifestyle modifications 2. Immediate BP drugs targeting < 140/90
1-2 risk factors	1. Lifestyle modifications 2. No BP intervention	1. Lifestyle modifications for several weeks 2. Then add BP drugs targeting < 140/90	1. Lifestyle modifications for several weeks 2. Then add BP drugs targeting < 140/90	1. Lifestyle modifications 2. Immediate BP drugs targeting < 140/90
≥ 3 risk factors	1. Lifestyle modifications 2. No BP intervention	1. Lifestyle modifications for several weeks 2. Then add BP drugs targeting < 140/90	1. Lifestyle modifications 2. BP drugs targeting < 140/90	1. Lifestyle modifications 2. Immediate BP drugs targeting < 140/90
Organ damage, chronic kidney disease stage 3 or diabetes	1. Lifestyle modifications 2. No BP intervention	1. Lifestyle modifications 2. BP drugs targeting < 140/90	1. Lifestyle modifications 2. BP drugs targeting < 140/90	1. Lifestyle modifications 2. Immediate BP drugs targeting < 140/90
Symptomatic cardiovascular disease, chronic kidney disease stage ≥ 4 or diabetes with organ damage / risk factors	1. Lifestyle modifications 2. No BP intervention	1. Lifestyle modifications 2. BP drugs targeting < 140(90	1. Lifestyle modifications 2. BP drugs targeting < 140(90	1. Lifestyle modifications 2. Immediate BP drugs targeting < 140(90

Note: BP = blood pressure

2.2.5 NADPH oxidase-derived superoxide anion production in hypertension

Although essential hypertension is known as a major risk factor for CAD and its underlying process of atherosclerosis (Alexander, 1995; Hajjar et al., 2006; Mancia et al., 2013; Perk et al., 2012), the mechanisms that link this association are not fully understood (Li & Chen, 2004).

Since their discovery, ROS have been of increasing interest due to their important regulatory role in physiological and pathophysiological conditions (Frazziano, Champion & Pagano, 2012). Physiologically, ROS at normal levels act as signaling molecules to maintain vascular integrity by regulating endothelial function and vascular contraction-relaxation. However, under pathological conditions, increased ROS production leads to increased contractility, vascular smooth muscle cell growth, monocyte invasion, lipid peroxidation, inflammation, increased deposition of extracellular matrix proteins, and endothelial dysfunction (Touyz, 2004). Thus, ROS influence cardiovascular physiology and are strongly implicated in pathological pathways leading to hypertension (Datla & Griendling, 2010).

To date, a growing body of animal and human studies suggests that NADPH oxidase and the resulting production of ROS, particularly superoxide anions, are involved in the pathogenesis of hypertension and the initiation of atherosclerosis in hypertension, respectively (Cai & Harrison, 2000; Touyz, 2004).

2.2.5.1 Animal studies

2.2.5.1.1 Vascular NADPH oxidase-derived superoxide anion production

Several studies in different animal models of hypertension have established the role of *vascular* NADPH oxidases in the development and progression of hypertension.

Angiotensin II-induced hypertension

Ang II is the main effector of the renin-angiotensin system and is implicated in the pathogenesis of hypertension (Li, Wheatcroft, Fan, Kearney & Shah, 2004) by affecting the cardiovascular system (e.g. vasoconstriction, induction of vascular smooth muscle cell growth, modulation of myocardial hypertrophy and fibrosis; Lee, Böhm, Paul & Ganten, 1993; Rajagopalan et al., 1996). Furthermore, certain forms of hypertension have been shown to be associated with higher circulating levels of Ang II (Lee et al., 1993; Rajagopalan et al.,

1996). Moreover, Ang II is the main agonist for oxidase activation in the vasculature (Brandes & Kreuzer, 2005).

Evidence from various animal models with altered NADPH oxidase subunit expression supports the role of NADPH oxidase and NADPH oxidase-derived superoxide anions in the pathogenesis of Ang II-induced acute and chronic hypertension (Cai & Harrison, 2000; Paravicini & Touyz, 2008).

In Ang II-induced acute hypertension, higher protein levels of NADPH oxidase subunits $p67^{phox}$, $gp91^{phox}$ (Cifuentes, Rey, Carretero & Pagano, 2000; Rey, Cifuentes, Kiarash, Quinn & Pagano, 2001), $p47^{phox}$ (Landmesser et al., 2002; Li et al., 2004; Wang et al., 2001), or $p22^{phox}$ (Modlinger et al., 2006) seem to be required for NADPH oxidase-derived production of superoxide anion and BP response to Ang II. Indeed, in vascular tissue of the aorta of Ang II-induced acute hypertensive mice, an increased NADPH oxidase-derived superoxide anion production accompanied by increased expression of $p67^{phox}$ and $gp91^{phox}$ subunits was reported (Cifuentes et al., 2000; Rey et al., 2001). In $p47^{phox}$ deficient mice, the hypertensive response to acute Ang II infusion was markedly blunted, and no increase of NADPH oxidase-derived superoxide anion production in aortic endothelial cells was found compared to Ang II-induced hypertensive wild-type mice (Landmesser et al., 2002; Li et al., 2004). In addition, in $gp91^{phox}$ knockout mice, basal BP was lower than in wild-type mice but Ang II infusion caused analogous increases in BP in both groups. Similar to $p47^{phox}$ deficient mice, Ang II infusion did not increase NADPH oxidase-derived aortic superoxide anion production in $gp91^{phox}$ knockout mice, whereas in the vascular tissue of the aorta of wild-type mice, the production was twofold higher (Wang et al., 2001). A further study showed that Ang II infusion causes a progressive increase in mean arterial blood pressure (MAP) in rats concomitantly with increased expression of $p22^{phox}$, renal NADPH oxidase activity, and NADPH oxidase-derived superoxide anion production (Modlinger et al., 2006).

In contrast to Ang II-induced acute hypertension, in Ang II-induced chronic hypertension, higher expression of NADPH oxidase subunit Nox1 (Dikalova et al., 2005; Gavazzi et al., 2006; Matsuno et al., 2005) but not $gp91^{phox}$ (Touyz et al., 2005) seems to play an important role in NADPH oxidase-derived superoxide anion production and BP elevation in response to Ang II (Gavazzi et al., 2006; Matsuno et al., 2005) as well as in the development of cardiovascular pathologies (Dikalova et al., 2005). Studies in vascular tissue of the aorta of Nox1 knockout mice revealed a reduction in both NADPH oxidase-derived superoxide anion production and BP elevation in response to Ang II (Gavazzi et al., 2006; Matsuno et al.,

2005). Furthermore, transgenic mice overexpressing Nox1 in VSMCs showed higher basal superoxide anion levels but similar basal BP compared to wild-type mice. In response to Ang II infusion, the NADPH oxidase-derived superoxide anion production and the hypertensive response was potentiated in transgenic mice (Dikalova et al., 2005). In contrast, a study of $gp91^{phox}$ deficient mice with Ang II-dependent chronic hypertension demonstrated that the development of hypertension is not affected by $gp91^{phox}$ and the associated reduction of NADPH oxidase-derived superoxide anion production tested in vascular cardiac, aortic, and renal tissue (Touyz et al., 2005).

Notably, although it has been shown that norepinephrine infusion produces a similar degree of hypertension to Ang II, it does not seem to be associated with an increase in NADPH oxidase-derived superoxide anion production (Rajagopalan et al., 1996). This suggests that superoxide anions are especially involved in certain forms of hypertension, which are associated with an increased vascular action of Ang II (Landmesser & Harrison, 2001). However, NADPH oxidase-derived superoxide anions also seem to be involved in the pathogenesis of hypertension independently of direct Ang II actions (Paravicini & Touyz, 2008). This is evidenced by findings that NADPH oxidase activity and NADPH oxidase-derived superoxide anion production is increased and that treatment with agents that inhibit NADPH oxidase-derived superoxide anion production reduces BP elevation in deoxycorticosterone acetate (DOCA)-salt, renovascular, and genetic hypertension (Jung, Schreiber, Geiger, Pedrazzini, Busse & Brandes, 2004; Zalba et al., 2000). Results of these studies are summarized in more detail in the following.

Deoxycorticosterone acetate-salt and renovascular hypertension

In vascular segments of the aorta of DOCA-salt hypertensive rats (i.e. a hypertension model characterized by a depressed plasma renin activity), BP and superoxide anion production were increased compared to controls (Beswick, Zhang, Marable, Catravas, Hill & Webb, 2001a; Callera et al., 2003; Somers, Mavromatis, Galis & Harrison, 2000). This increase in superoxide anion production was associated with an increase in NADPH oxidase activity (Beswick, Dorrance, Leite & Webb, 2001b; Wu, Millette, Wu & de Champlain, 2001) and mRNA levels for the NADPH oxidase subunit $p22^{phox}$ (Beswick et al., 2001). Furthermore, a long-term inhibition of NADPH oxidase significantly decreases superoxide anion production and BP in DOCA-salt rats, suggesting that an increased NADPH oxidase activity is likely

responsible for increased superoxide anion production and possibly contributes to increased BP (Beswick et al., 2001a).

In vascular segments of the aorta of the 2-kidney 1-clip (2K-1C) model of renovascular hypertension (i.e. a hypertension model characterized by a high activation of the renin-angiotensin system), an overactivity of the NADPH oxidase accompanied by an increased NADPH oxidase-derived superoxide anion production was reported. The addition of a PKC inhibitor reduced the observed NADPH oxidase-derived superoxide anion overproduction and improved endothelial dysfunction, indicating that the increased superoxide anion production is likely secondary to a PKC-mediated activation of NADPH-dependent oxidase (Heitzer et al., 1999). In $gp91^{phox}$ knockout mice, renovascular hypertension induced by 2K-1C was less severe than in wild-type mice. Furthermore, in wild-type mice with renovascular hypertension, endothelial dysfunction was due to an increased NADPH oxidase-derived superoxide anion production with associated reduction in NO bioavailability as measured in vascular tissue of the aorta. These findings suggest that superoxide anions produced by an endothelial $gp91^{phox}$-containing NADPH oxidase mediate endothelial dysfunction in renovascular hypertension by scavenging endothelium-derived NO (Jung et al., 2004).

Genetic hypertension

In VSMCs of spontaneously hypertensive rats (SHR), an animal model of essential hypertension, an increased NADPH oxidase-derived superoxide anion production associated with an upregulated $p22^{phox}$ mRNA expression compared to age-matched Wistar Kyoto rats was reported. The observed overproduction of NADPH oxidase-derived superoxide anion production was further associated with a reduction in NO bioavailability, and thus endothelial dysfunction. These abnormalities were normalized in SHR treated with an Ang II type 1 receptor blocker. These results also indicate on the one hand a possible contribution of NADPH oxidase-derived superoxide anion production in endothelial dysfunction and on the other hand an important role of Ang II in the upregulation of NADPH oxidase (Zalba et al., 2000). Furthermore, in VSMCs of SHR, the presence of several polymorphisms in the promoter region of the $p22^{phox}$ was observed, providing a potential explanation for the upregulated NADPH oxidase activity in the vessel wall of SHR (Zalba, San José, Beaumont, Fortuño, Fortuño & Diez, 2001). In stroke-prone SHR, a genetic model of severe hypertension and cerebral stroke, the mRNA levels of Nox1 and Nox4 were higher compared to age-matched Wistar Kyoto rats, whereas mRNA expressions of $gp91^{phox}$ and $p22^{phox}$ were

comparable. Treatment with Ang II type 1 receptor blocker decreased BP and normalized the increased expression of Nox1 and Nox4 and associated superoxide anion production. These findings indicate that both Ang II and chronic hypertension upregulate NADPH oxidase subunits Nox1 and Nox4, which may contribute to the development of hypertensive vascular injury (Akasaki et al., 2006).

It should be noted that one important mechanism by which enhanced NADPH oxidase-derived superoxide anion production may contribute to the development of hypertension and the pathogenesis of atherosclerosis in hypertension is the inactivation of the endothelium-derived NO (Landmesser & Harrison, 2001). A reduced vascular NO bioavailability leads to a increase in BP, as reported in studies of endothelial nitric oxide synthase (NOS) knockout mice (Huang et al., 1995; Kojda et al., 1999) and rats (Baylis, Mitruka & Deng, 1992) after a blockade of NOS. Furthermore, studies in SHR have shown that an enhanced production of NADPH oxidase-derived superoxide anions causes a loss of NO bioavailability with associated endothelial dysfunction, suggesting that a diminished NO bioavailability, secondary to an increased NADPH oxidase-derived superoxide anion production, may play a critical role in endothelial dysfunction and thus promote atherosclerosis in essential hypertension (Grunfeld et al., 1995; Tschudi, Mesaros, Lüscher & Malinski, 1996; Schnackenberg, Welch & Wilcox, 1998; Zalba et al., 2001; Zalba et al., 2000).

2.2.5.1.2 Phagocytic NADPH oxidase-derived superoxide anion production

Although phagocytic cells (i.e. leukocytes such as neutrophils and monocytes / macrophages) of the innate immune system have been shown to contribute to hypertension (Harrison, 2014), few animal studies have examined the role of NADPH oxidase-derived superoxide anion production in these cells under hypertensive conditions (Mazor et al., 2007). However, there is evidence suggesting that phagocytic cells seem to contribute to the development of hypertension through an increased *phagocytic* NADPH oxidase-derived superoxide anion production (Maeda, Yasunari, Sato, Yoshikawa & Inoue, 2003; Mazor, Kristal, Cohen-Mazor, Yagil, Yagil & Sela, 2007; Schmid-Schönbein, Seiffge, DeLano, Shen & Zweifach, 1991; Sela, Mazor, Amsalam, Yagil, Yagil & Kristal, 2004) activated by TNF-α (Mazor et al., 2010). Indeed, SHR showed a significantly higher NADPH oxidase-derived superoxide anion production of circulating leukocytes compared to controls (Schmid-Schönbein et al., 1991; Maeda et al., 2003). This increased superoxide anion production was further associated with

an enhanced expression of PKC and $p47^{phox}$ (Maeda et al., 2003). Furthermore, a study comparing Sabra hypertension-resistant rats (SBN/y; salt-resistant) and Sabra hypertension-prone rats (SBH/y; salt-sensitive) before and during salt-loading (i.e. induction of hypertension) reported a higher prevalence of circulating polymorphonuclear leukocytes, with an associated higher rate of NADPH oxidase-derived superoxide anion production in SBH/y under both conditions (Sela, Mazor, Amsalam, Yagil, Yagil & Kristal, 2004). In addition, the inhibition of NADPH oxidase activity reduced the development of hypertension and decreased the rate of superoxide anion production in SBH/y (Mazor, Kristal, Cohen-Mazor, Yagil, Yagil & Sela, 2007). A subsequent study further analyzed TNF-α levels as a potential activating agent of NADPH oxidase in circulating polymorphonuclear leukocytes. The authors found higher TNF-α levels in SBH/y before and during salt-induced hypertension compared to SBN/y. The inhibition of NADPH oxidase activity significantly reduced these levels in SBH/y but not in SBN/y (Mazor et al., 2010).

2.2.5.2 Human studies

Despite the abundance of animal data implying an important role of *vascular* and *phagocytic* NADPH oxidase-derived superoxide anion production in the development of hypertension, findings in human hypertension are less convincing (Paravicini & Touyz, 2008; Touyz & Briones, 2011). This may be due to multiple factors, such as (1) complex regulation of ROS and redox signaling in the vascular system and (2) challenges related to direct measurements of NADPH oxidase-derived superoxide anion production in the clinical setting (Montezano, Dulak-Lis, Tsiropoulou, Harvey, Briones & Touyz, 2015). However, there is some evidence for an increased *vascular* and *phagocytic* NADPH oxidase-derived superoxide anion production in human hypertension.

2.2.5.2.1 Vascular NADPH oxidase-derived superoxide anion production

NADPH oxidase-derived superoxide anions of VSMCs may be implicated in the progression of hypertension. This is supported by findings in VSMCs from resistance arteries (i.e. blood vessels that form the major site of generation of vascular resistance) of patients with essential hypertension reporting higher levels of Ang II-induced generation of NADPH oxidase-derived superoxide anions compared to VSMCs from normotensive healthy controls (Touyz & Schiffrin, 2001; Touyz, Yao & Schiffrin, 2003; Touyz, Yao, Quinn, Pagano & Schiffrin,

2005). This increase was further associated with an enhanced activation of phospholipase D by Ang II, which may influence redox-sensitive pathways through phosphatidic acid and PKC (Touyz & Schiffrin, 2001).

2.2.5.2.2 Phagocytic NADPH oxidase-derived superoxide anion production

NADPH oxidase-derived superoxide anion production of phagocytic cells may play an important role in the development of hypertension and the pathogenesis of atherosclerosis in hypertension. This is evidenced by findings reporting an increased *phagocytic* NADPH oxidase-derived superoxide anion production and associated reduction in NO bioavailabilty in hypertension (Fortuño et al., 2004; Mehta, Lopez, Chen & Cox, 1994), which may contribute to endothelial dysfunction as an initial step in atherosclerosis (Ross, 1999).

In partially treated individuals with essential hypertension, NADPH oxidase-derived superoxide anion production in neutrophils (Mehta et al., 1994) and PMA (i.e. an activating agent of *phagocytic* NADPH oxidase in peripheral blood mononuclear cells (PBMCs))-simulated PBMCs (i.e. monocytes and lymphocytes) were higher, whereas NOS activity (Mehta et al., 1994) and serum NO concentration (Fortuño et al., 2004) were lower as compared to normotensive controls. Moreover, in PMA-stimulated PBMCs of hypertensive individuals, NADPH oxidase-derived superoxide anion production in response to an *in vitro* infusion of Ang II was significantly higher than in normotensives (Fortuño et al., 2004). The importance of NADPH oxidase-derived superoxide anions in contributing to hypertension was further reflected in the existence of a $p22^{phox}$ polymorphism in patients with essential hypertension. In partially treated participants with essential hypertension, the prevalence of the C242T polymorphism – the human gene encoding $p22^{phox}$ – was higher as compared to normotensive controls and remained independently associated with hypertension after adjusting for cardiovascular risk factors such as age, BMI, lipid profile, fibrinogen, and von Willebrand factor. In addition, hypertensive participants with the C242T polymorphism showed a significantly increased PMA-stimulated NADPH oxidase-derived superoxide anion production of PBMCs compared to controls (Moreno et al., 2006). In a further study in individuals with untreated essential hypertension, ROS formation by mononuclear cells but not polymorphonuclear leukocytes was positively associated with carotid artery IMT, as a vague indicator for atherosclerosis severity (Watanabe et al., 2006). Moreover, a very recent cross-sectional study in treated hypertensive men and women showed that superoxide anion production in PMA-stimulated PBMCs was increased in hypertensives with LVH compared

to those without LVH and normotensives. Superoxide anion production was also higher in hypertensive individuals without LVH than in normotensives. Furthermore, NADPH oxidase-derived superoxide anion production was independently associated with left ventricular mass index (Moreno et al., 2014).

2.3 Coronary artery disease

CAD is the most common type of heart disease and is considered as the global leading public health burden (Weber & Noels, 2011; WHO, 2008). In 2010, CAD caused 7 million deaths worldwide, representing an increase of 35% since 1990 (Wong, 2014). Furthermore, CAD is the single most common cause of death in Europe, with 1.8 million deaths, or 20% of deaths, annually (Nichols et al., 2014). According to the current Heart Disease and Stroke Statistics, international CAD death rates (per 100'000 population) vary 15-fold in men aged between 35 and 75 years (ranging from 47 in France to 718.1 in the Ukraine) and approximately 30-fold in age-matched women (ranging from 10.5 in France to 294.7 in the Ukraine). In addition, each year, an estimated 635'000 Americans suffer from a new coronary attack and a further 300'000 have a recurrent attack (Mozaffarian et al., 2015). Although death rates have fallen from 1968 to the present as a result of improved medical care (Mozzafarian et al., 2015; Nichols et al., 2012), it is predicted that CAD will remain the leading cause of death worldwide until 2030 (Mathers & Loncar, 2006).

Atherosclerosis is the single most important contributor to CAD (Libby, 2002). Therefore, the following section describes atherosclerotic disease and its pathogenesis and further elucidates the role of monocyte / macrophages within this process.

2.3.1 Atherosclerosis

Atherosclerosis is the underlying pathophysiological process of CAD (Libby & Theroux, 2005) and was previously considered as a cholesterol storage disease that obstructs arteries (Libby, Okamoto, Rocha & Folco, 2010). However, over the past few years, substantial advances in basic and experimental science have shown that atherosclerotic lesions are highly specific, dynamic, cellular, and molecular inflammatory responses (Farzaneh-Far, Rudd & Weissberg, 2001; Libby & Theroux, 2005). Thus, the importance attributed to inflammation in atherosclerosis has greatly increased (Libby & Theroux, 2005). As a result, atherosclerosis is now considered as a progressive chronic inflammatory process of arterial wall thickening and is further characterized by intense immunological activity (Hansson & Libby, 2006). Because atherosclerosis promotes vascular accumulation of lipids and fibrous elements in the inner layer of the arterial wall (i.e. intima) of many medium-sized and large arteries (particularly where the vessels divide), leading to remodeling of peripheral blood vessels and

impairment of coronary vessel functioning and blood flow dynamics (Forstegard, 2013; Gianaros & Sheu, 2009; Libby, 2002; Libby & Theroux, 2005; Ross, 1999), atherosclerosis poses an increasing threat to human health (Hansson & Libby, 2006). Indeed, atherosclerosis accounts for one fifth of all deaths worldwide (Ghattas et al., 2013).

2.3.1.1 Pathogenesis of atherosclerosis

Various hypotheses have been postulated to explain the pathophysiological processes of atherosclerosis, including the '*response-to-injury'* hypothesis (Ross, 1999), the '*response-to-retention*' hypothesis (Williams & Tabas, 1995) and the '*oxidation hypothesis'* (Witztum, 1994). However, the '*response-to-injury'* hypothesis has the largest amount of scientific support (Gudimetla & Kusumoto, 2004) and represents a theoretical feature of our experimental design. Therefore, this hypothesis will be described in the following.

2.3.1.1.1 Response-to-injury hypothesis

The '*response-to-injury'* hypothesis was first proposed by Virchow in 1856, and mainly driven forward by Ross and colleagues (Ross & Glomset. 1973; Ross, Glomset & Harker, 1977; Ross, 1986; Ross, 1999). The hypothesis points out that the vascular endothelium encounters a number of insults that evoke an inflammatory response. If the insults are not eliminated, a chronic inflammatory state develops, resulting in an early atherosclerotic lesion. Left untreated, this early atherosclerotic lesion can progress to an advanced, complicated lesion (Millar, 2011; Ross, 1999).

According to Ross (1999), the '*response-to-injury'* hypothesis of atherosclerosis proposes four stages of atherosclerosis: (I) endothelial dysfunction, (II) formation of fatty streaks, (III) formation of an advanced, complicated lesion, and (IV) unstable fibrous plaques. Every stage represents a characteristic lesion of atherosclerosis and a different stage in a chronic inflammatory process in the artery (Ross, 1999).

I Endothelial dysfunction

Endothelial dysfunction is proposed as the initial step of atherosclerosis (Ross, 1999). The endothelium is an active, dynamic tissue. As a continuous layer of cells, the endothelium separates blood from the vessel wall and controls many important functions, including maintenance of blood circulation and fluidity as well as regulation of vascular tone, coagulation, and inflammatory responses (Gonzalez & Selwyn, 2003). Cardiovascular risk factors such as elevated LDL or hypertension profoundly affect many of the healthy functions of the endothelium (Gonzalez & Selwyn, 2003) and lead to compensatory responses that alter the normal homeostatic properties of the endothelium (Ross, 1999). These changes include increased endothelial adhesiveness and permeability to plasma lipoproteins, leukocytes and platelets, up-regulation of leukocyte and endothelial adhesion molecules, and migration of leukocytes into the intima (Ross, 1999).

Notably, the exact nature of the link between cardiovascular risk factors and endothelial dysfunction is still unclear (Cullen, Rauterberg & Lorkowski, 2005).

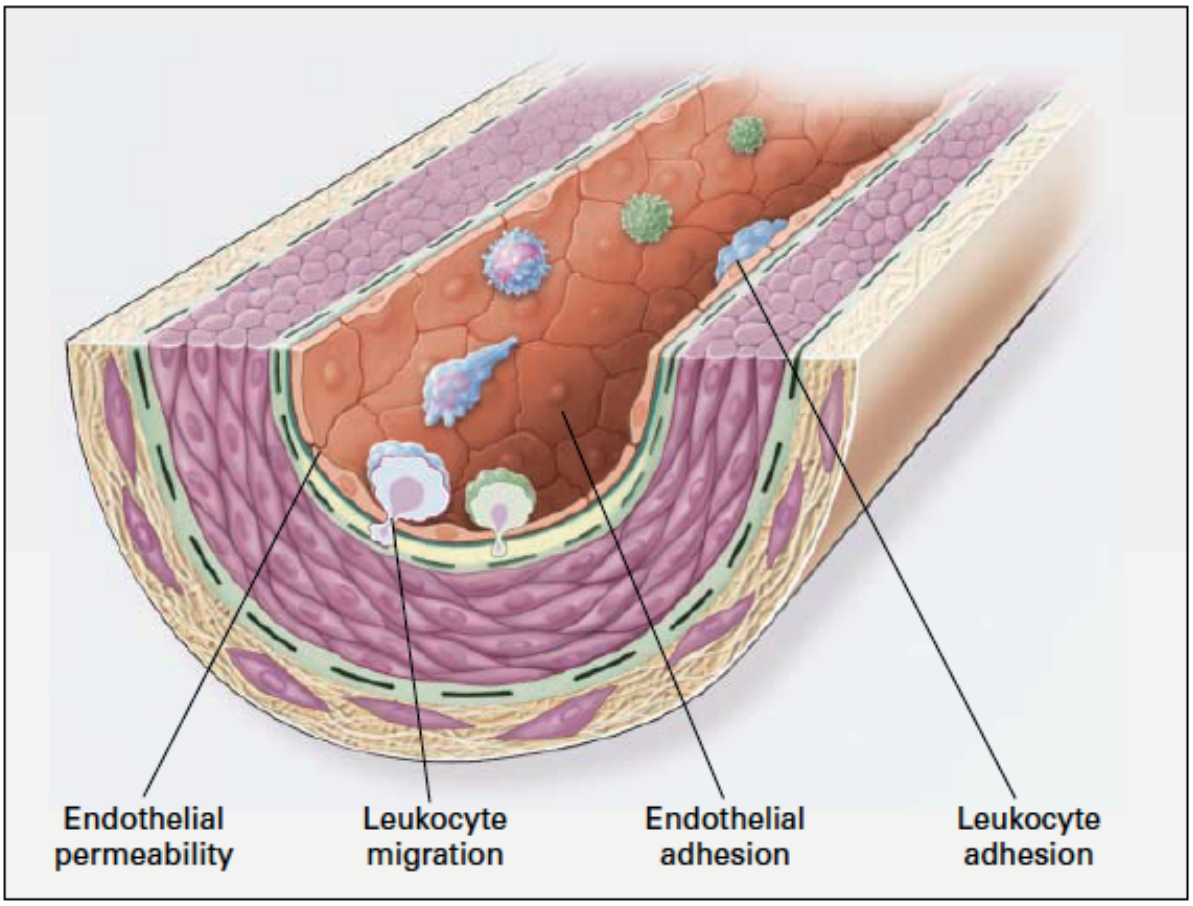

Figure 5: Endothelial dysfunction in atherosclerosis. Ross (1999).

II Formation of fatty streaks

Fatty streaks represent the earliest type of lesion in the process of atherogenesis. As a pure inflammatory lesion, fatty streaks consist, among others, of lipid-laden monocyte-derived macrophages (foam cells) and T lymphocytes (Ross, 1999).

Due to increased endothelial permeability, LDL particles infiltrate into the intima, where they can undergo progressive oxidation and be internalized by macrophages (Libby, 2002; Ross, 1999). In addition, lipid peroxidation products generated during LDL oxidation are chemotactic for other monocytes and T cells (Napoli, D'Armiento, Mancini, Postiglione, Witztum, Palumbo & Palinski, 1987) and can enhance the expression of genes for macrophage colony-stimulating factor and monocyte chemotactic protein derived from endothelial cells (Ross, 1999). If this inflammatory reaction continues, it stimulates the migration of smooth muscle cells. This migration further thickens the artery wall, resulting in an intermediate lesion (Ross, 1999).

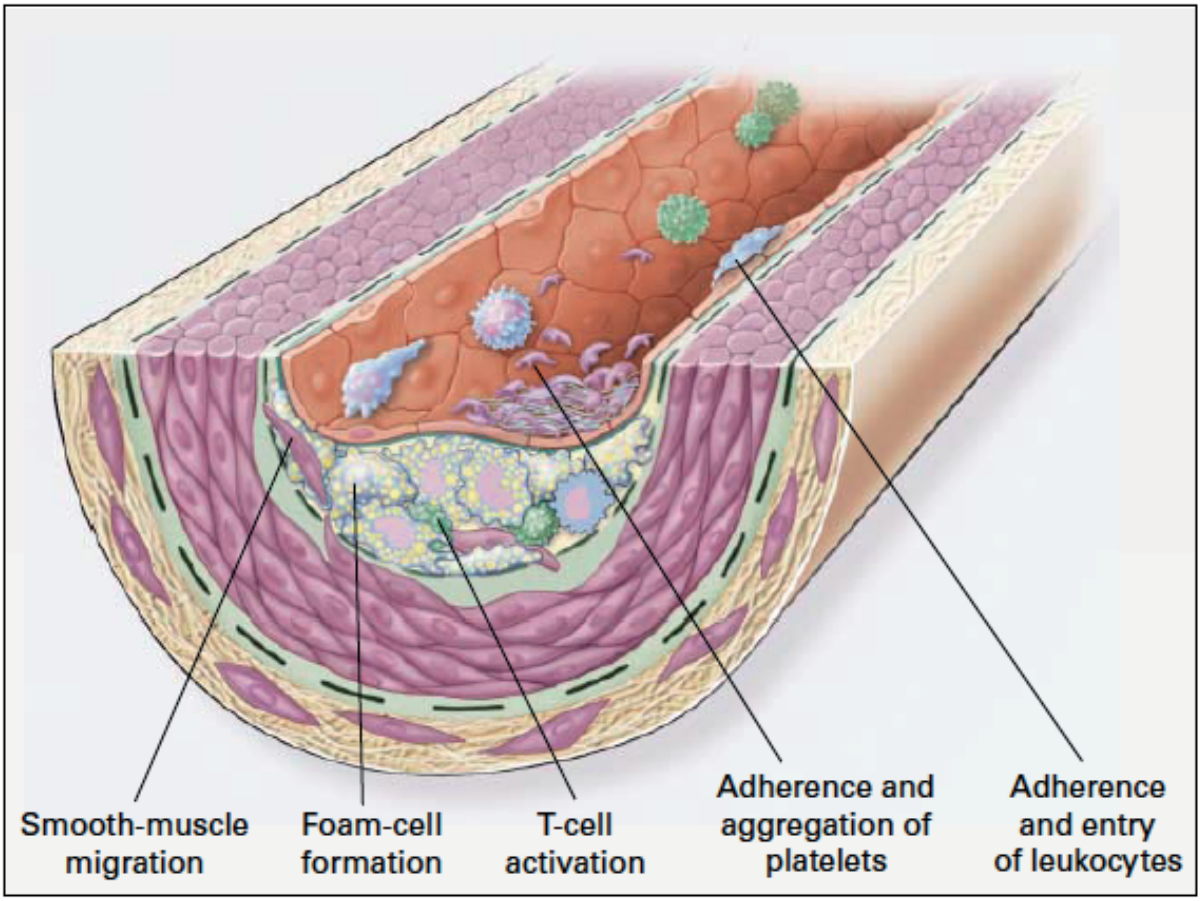

Figure 6: Fatty streak formation in atherosclerosis. Ross (1999).

III Formation of advanced, complicated lesion

Continued inflammation results in increased influx of macrophages and lymphocytes from the blood into the lesion. Activation of these cells leads to the release of hydrolytic enzymes, cytokines, chemokines, and growth factors, resulting in an enlargement and restructuring of the lesion, which subsequently becomes covered by a fibrous cap (Ross, 1999). The fibrous cap covers a mixture of leukocytes, lipids, and debris (Ross, 1999) released from dying macrophages and leads to the formation of a prothrombotic necrotic core (Moore, Sheedy & Fisher, 2013). This inflammatory response is termed an advanced, complicated lesion. Existing lesions further expand at their shoulders by means of continued leukocyte adhesion and entry, and thus alter the blood flow (Ross, 1999).

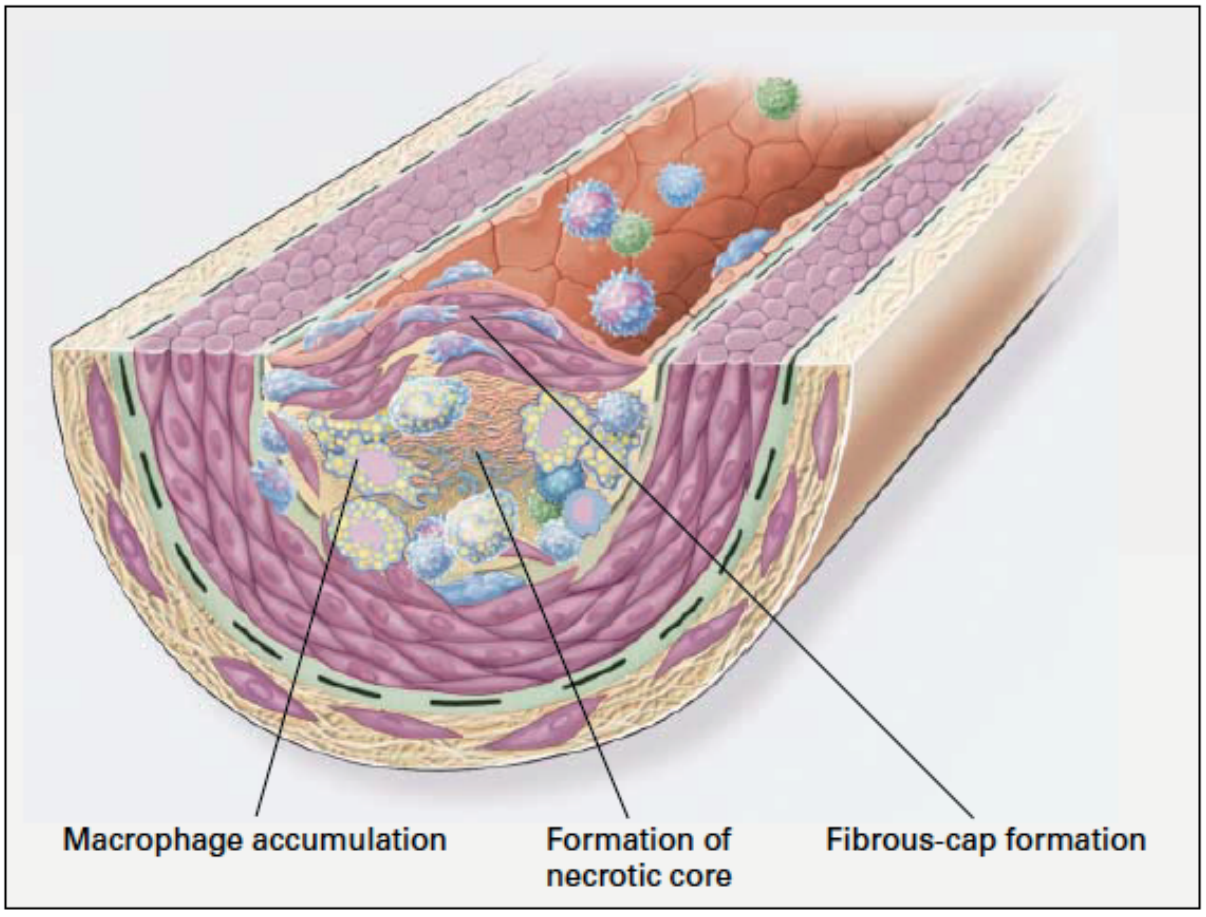

Figure 7: Formation of advanced, complicated lesion of atherosclerosis. Ross (1999).

IV Unstable fibrous plaques

Continued influx and activation of macrophages and the resulting release of proteolytic enzymes – which may cause hemorrhage from the vasa vasorum or from the lumen of the artery – lead to thinning of the fibrous cap. There, rupture of the fibrous cap or ulceration of the fibrous plaque can lead to thrombosis. Notably, plaque rupture and thrombosis are considerable complications of advanced lesions that lead to CAD (Ross. 1999).

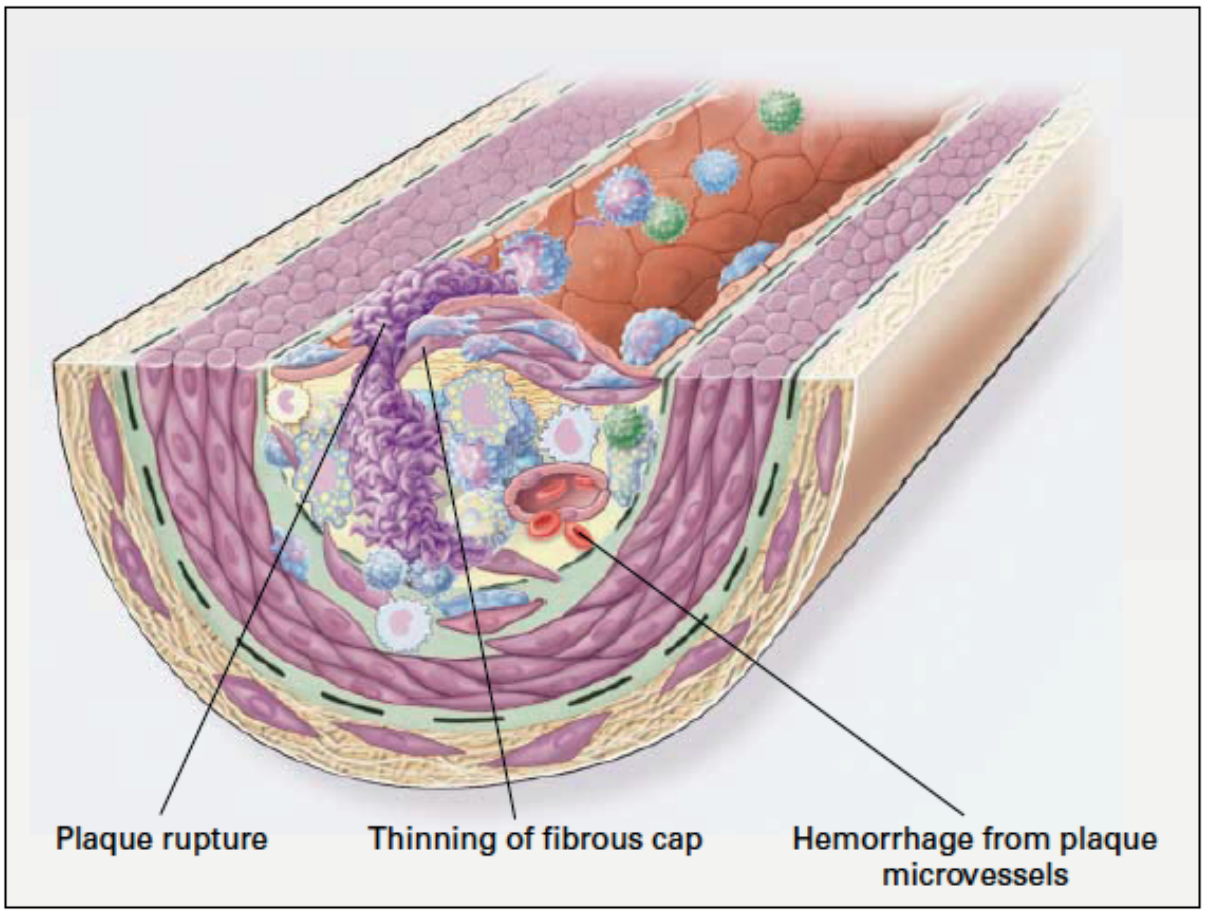

Figure 8: Unstable fibrous plaque in atherosclerosis. Ross (1999).

2.3.1.2 The role of monocytes / macrophages

Monocytes and monocyte-derived inflammatory macrophages are key cells in the initiation and progression of atherosclerosis (Ghattas et al., 2013; Ley et al., 2011; Moore & Tabas, 2011). Moreover, monocyte-derived inflammatory macrophages represent the main component of atherosclerotic plaques (Gui, Shimokado, Sun, Akasaka & Muragaki, 2012).

2.3.1.2.1 Monocyte entry

In addition to the endothelial dysfunction described above, a further early inflammatory response in atherogenesis is the activation of overlying endothelial cells as a result of subendothelial LDL particles accumulation (Cullen et al., 2005; Moore & Tabas, 2011). Activated endothelial cells express a number of proinflammatory genes such as vascular cell adhesion molecules 1 and chemokines (Mestas & Ley, 2008; Moore & Tabas, 2011). Vascular cell adhesion molecule 1 is needed for the slow rolling of monocytes, and therefore plays a crucial role in the initial steps of monocyte recruitment to atherosclerotic lesions. Chemokines, particularly C-C motif chemokine ligand 2 (CCL2) and C-C motif chemokine ligand 5 (CCL5), in turn, affect the recruitment of monocytes to the vessel wall (Mestas & Ley, 2008). They interact with receptors on monocytes and promote the infiltration of blood monocytes into the intima (Moore & Tabas, 2011). The process of monocyte migration from circulating blood into the connective tissue (e.g. subendothelial connective tissue of the intima) is called diapedesis (Gui et al., 2012; Moore & Tabas, 2011). Notably, animal studies have shown that the prevention of monocyte entry by blocking chemokines or their receptors prevents or retards atherogenesis (Moore & Tabas, 2011).

2.3.1.2.2 Monocyte differentiation into macrophages

In the intima, monocytes differentiate into M1 or M2 macrophages based on stimulation by macrophage colony-stimulating factor (Ilhan & Kalkanli, 2015). Notably, M1 and M2 play opposite roles during inflammation (Gui et al., 2011): Whereas M1 macrophages promote inflammation resulting in plaque vulnerability, M2 macrophages promote resolution of inflammation and may increase plaque stability (Gui et al., 2011; Ilhan & Kalkanli, 2015). In the past years, there has been great interest in the roles of M1 and M2 macrophages in atherosclerotic lesions, but no definitive conclusions have yet emerged from these studies (Gui et al., 2012; Johnson & Newby, 2009; Moore & Tabas, 2011).

2.3.1.2.3 Foam cell formation

Monocyte-derived inflammatory macrophages in the intima exhibit enhanced expression of pattern recognition receptors, including scavenger receptors (Singh, Mengi, Xu, Arneja & Dhalla, 2002). Scavenger receptors allow the uptake of accumulated normal and oxidized LDL and thus contribute to the formation of foam cells (Moore et al., 2013; Singh et al.,

2002; Woollard & Geissmann, 2010). Lipid-laden foam cells are specific macrophages (Moore et al., 2013), which release additional extracellular matrix components that further support lipoprotein retention within the sub-endothelium. Furthermore, they produce chemokines, cytokines, proteases, growth factors, bioreactive lipids, and angiogenic factors that perpetuate the inflammatory process and lead to the migration of smooth muscle cells into the intima. Moreover, activated macrophages in developing plaques produce ROS, which cause cell apoptosis and lipoprotein oxidation (Wilson, 2010). This inflammatory response contributes to plaque formation and promotes disease progression (Moore et al., 2013).

2.3.1.2.4 Macrophages in advanced atherosclerosis

In atherosclerosis, as a non-resolving inflammatory condition, monocytes continue to enter into the intima, convert into macrophages, and lead to the progression of atherosclerotic plaques. Subsequently, in advanced plaques, macrophages contribute to changes in plaque morphology, formation of the necrotic core (arising from the combination of apoptosis of macrophages and the phagocytic clearance of the apoptotic cells in advanced plaques), and thinning of the fibrous cap (Gui et al., 2012; Moore & Tabas, 2011). In addition, macrophages release proteolytic enzymes that degrade matrix components and the protective fibrous cap, leading to plaque destabilization and increased risk of plaque rupture and thrombosis. Notably, the number of macrophages within plaques is associated with reduced plaque stability. Therefore, plaques tend to rupture at sites of increased macrophage content (Wilson, 2010).

In sum, monocytes and monocyte-derived inflammatory macrophages play a pivotal role in every stage of atherosclerosis, from its initiation to progression and complications (Ley et al., 2011).

2.3.1.3 Diagnosis of atherosclerosis

The diagnosis of atherosclerosis in its early stages is required to prevent the development and progression of atherosclerotic plaques as a sign of advanced atherosclerosis (Latifoglu, Sahan, Kara & Günes, 2007; Mercuri, 1995).
Several invasive (e.g. coronary angiography, computed tomography coronary artery calcium testing method) and noninvasive (e.g. myocardial perfusion imaging, single-photon emission

computed tomography, positron emission tomography, cardiac computed tomography) diagnostic modalities are available to identify signs of atherosclerosis (Kasliwal, Bansal, Densai & Sharma, 2014; Mercuri, 1995), but the majority are costly and / or unable to reliably detect early atherosclerosis (Kasliwal et al., 2014). In contrast, the assessment of carotid IMT and / or carotid plaque – a surrogate marker of atherosclerosis (Groot et al., 2004) – represents a noninvasive and inexpensive procedure that reliably detects early stages of atherosclerosis (Darabian, Hormuz, Latif, Pahlevan & Budoff, 2013; Kasliwal et al., 2014; Onut, Balanescu, Constantinescu, Calmac, Marinescu & Dorobantu, 2012).

2.3.1.3.1 Carotid intima-media thickness and carotid plaque assessment

Carotid IMT is measured between the intimal-luminal and the medial-adventitial interfaces of the carotid artery wall (Naqvi & Lee, 2014) and can be reliably determined in vivo (Onut et al., 2012) by B-mode (bright-mode) ultrasound (Darabian et al., 2013; Onut et al., 2012; Simon, Gariepy, Chironi, Megnien & Levenson, 2002). Carotid ultrasound represents a readily available, inexpensive, fast, precise, reproducible, and widely used noninvasive procedure for identifying and quantifying subclinical atherosclerosis. It provides information about common carotid (CCA), bifurcation, and internal and external carotid arteries (Darabian et al., 2012; Simon et al., 2002).

For ultrasound imaging in carotid IMT, transducers with a frequency of at least 7 MHz to maximum 15 MHz are required (Darabian et al., 2013). They produce acoustic or sound waves for an accurate diagnosis with improved image quality (Kasliwal et al., 2014). In general, the far wall (i.e. the farthest carotid wall to the transducer) of the left and right CCA (multiple measurements of mean and maximum values) is utilized for the measurement of the carotid IMT (Darabian et al., 2013; Onut et al., 2012) due to the high precision and reproducibility (Navqi & Lee, 2014; Simon et al., 2002). Carotid IMT above the 75th percentile or absolute thickness more than 1.0 mm are considered abnormal (Darabian et al., 2013; Naqvi & Lee, 2014). A carotid plaque has been defined either as a focal thickening that encroaches into the arterial lumen by at least 0.5 mm or 50% of the surrounding IMT value or an IMT greater than 1.5 mm measured from the media-adventia interface to the intima-lumen interface (Naqvi & Lee, 2014; Onut et al., 2012; Touboul et al., 2012). Notably, carotid IMT varies with age, gender, and ethnicity (Onut et al., 2012).

A number of clinical studies demonstrated an association between carotid IMT and future coronary events (Darabian et al., 2013; Navqi & Lee, 2014; Onut et al., 2012). A systematic review and meta-analysis of eight observational studies with general population-based samples (a total of 37'197 participants followed for a mean of 5.5 years) showed that with an absolute carotid IMT difference of 0.1 mm, the future risk of myocardial infarction (MI) increases by 10- 15%, whereas the risk for stroke increases by 13% to 18 % (Lorenz, Markus, Bots, Rosvall & Sitzer, 2007). Furthermore, carotid IMT has been shown to be independently associated with the incidence of cardiovascular events (Bots, Hoes, Koudstall, Hofman & Grobbee, 1997; Chembless, Heiss, Folsom, Rosamon, Szklo, Sharrett & Clegg, 1997: Lorenz, Schaefer, Steinmetz & Sitzer, 2010; Rosvall, Janzon, Berglund, Engström & Hedblad, 2005). Notably, a recent meta-analysis showed that the assessment of carotid plaque had a higher diagnostic accuracy for predicting future CAD events compared to carotid IMT (Inaba, Chen & Bergman, 2012). However, a combined carotid IMT and plaque assessment appears to be favorable as compared to either measure alone (Naqvi & Lee, 2014).

Despite the associations reported above, the current guidelines of the American College of Cardiology Foundation / American Heart Association do not recommend carotid IMT assessment as a screening method for atherosclerosis (Brott et al., 2011; Onut et al., 2012). This might be due to the limited value of carotid IMT for risk stratification of individuals in the general population (Lorenz et al., 2010).

2.3.2 Clinical manifestations of coronary artery disease

As a slowly developing chronic illness, CAD mainly results from an atheromatous narrowing and subsequent occlusion of the blood vessels that supply oxygenated blood to the heart (Gianaros & Sheu, 2009; Grech, 2003). Consequently, this vessel narrowing can lead to several clinical manifestations, including chest pain (angina pectoris), an inadequate ejection of blood from the heart (heart failure), irregular cardiac rhythms (arrhythmias), and acute coronary syndrome caused by an insufficient supply of blood to cardiac tissues (myocardial ischemia; Davies, 2001; Gianaros & Sheu, 2009).
Acute coronary syndromes – the most important clinical manifestations of CAD – will be briefly described in the following.

2.3.2.1 Acute coronary syndrome

Acute coronary syndrome (ACS) represents a major cause of morbidity and mortality in Western countries and is responsible for more than 2.5 million hospitalizations annually worldwide (Grech & Ramsdale, 2003).

ACS involves atherosclerotic plaque rupture and the subsequent thrombus formation, resulting in limitation or interruption of coronary blood flow (Nikolsky & Stone, 2007) and includes the clinical conditions of unstable angina pectoris and acute MI (Rentrop, 2000).

2.3.2.1.1 Unstable angina pectoris

Unstable angina pectoris (UAP) is the most threatening form of angina and should be distinguished from stable angina pectoris (chest pain that occurs over a long period with the same pattern of onset, duration, and intensity of symptoms) and Prinzmetal / variant angina pectoris (often occurs at rest, usually in response to spasm of a major coronary artery; Bucher & Johnson, 2014).

UAP is a clinical syndrome of discomfort in the chest (Davies, 2001). Due to a reduced blood flow to the heart muscle, UAP represents a critical phase of CAD (Hamm & Braunwald, 2000). Based on clinical experience and validated in several prospective studies (Hamm & Braunwald, 2000), Braunwald (1989) classified UAP into three groups according to the severity of clinical manifestation: (Class I) new onset of severe or accelerated angina without rest pain, (Class II) subacute UAP at rest (one or more episodes of angina at rest during the preceding month but not within the preceding 48 hours), and (Class III) acute UAP at rest (one or more episodes of angina at rest within the preceding 48 hours). Moreover, Braunwald (1989) defined clinical circumstances in which UAP occurs: (Class A) secondary UAP (develops in the presence of an extracardiac condition that intensifies myocardial ischemia), (Class B) primary UAP (develops in the absence of an extracardiac condition), and (Class C) postinfarction UAP (develops within the first two weeks after acute MI). Due to this heterogeneity of clinical manifestation, the prognosis of UAP is quite variable (Yeghiazarians, Braunstein, Askari & Stone, 2000).

It should be noted that UAP is usually a harbinger of acute MI, and is therefore often termed as preinfarction angina (Braunwald & Morrow, 2013).

2.3.2.1.2 Acute myocardial infarction

Acute MI represents one of the main manifestations of CAD (Bucher & Johnson, 2014; Mendis et al., 2010). Worldwide, more than 7 million people annually are estimated to have an acute MI (White & Chew, 2008), although the incidence of acute MI has decreased since the year 2000 (Moran et al., 2014; Yeh, Sidney, Chandra, Sorel, Selby & Go, 2010). Importantly, patients who survive an initial acute MI are at higher risk for a recurrent event (Mendis et al., 2010).

Acute MI is defined as irreversible myocardial cell death (necrosis) due to significant and sustained ischaemia (Mendis et al., 2011) and may be the first manifestation of atherosclerosis-related CAD (Thygesen, Alpert & White, 2007). The initial step of acute MI is the onset of myocardial ischaemia due to the imbalance between oxygen supply and demand. The process of myocardial ischaemia may be accompanied by possible unspecific ischaemic symptoms in the chest, upper extremity, or jaw, which usually last at least 20 minutes. An acute MI may occur with atypical symptoms such as palpitations or cardiac arrest, or even without symptoms (Thygesen et al., 2007) and its clinical presentation varies from a minor coronary event to life-threatening clinical situations or sudden death (Mendis et al., 2011).

Acute MI is generally classified into *ST-segment elevation acute MI* (STEMI; complete coronary occlusion) and *non-ST-segment elevation acute MI* (NSTEMI; transient thrombosis or incomplete coronary occlusion). The *ST-segment* refers to a characteristic section on a 12-lead electrocardiogram. Changes in this segment (e.g. elevation or depression) indicate the severity of an MI (Bucher & Johnson, 2014). Notably, most patients with NSTEMI were diagnosed as having UAP (Boersma, Mercado, Pldemans, Gardien, Vos & Simoons, 2003). The 30-day mortality rate is higher in patients with STEMI compared to those with NSTEMI (Kumar & Cannon, 2009), whereas the one-year death rate is higher in NSTEMI than in STEMI (McManus, Gore, Yarzebski, Spencer, Lessard & Goldberg, 2011). The observed higher long-term death rate in NSTEMI may result from the fact that patients with NSTEMI were older and had a greater burden of cardiovascular comorbidities (McManus et al., 2011).

Sudden cardiac death is a fatal and unpredicted consequence of acute MI that results from ischemia, which provokes lethal ventricular arrhythmias during the acute phase of MI (Bunch, Hohnloser, & Gersh, 2007).

2.3.3 Risk factors for coronary artery disease

The risk of a CAD event is multifactorial (Wilson, 2004). To promote understanding of this important disease, precursors of CAD have been extensively studied, and risk factors have been identified (Greenland et al., 2003) which are associated with an increased rate of CAD incidence (O'Donnell & Elosua, 2008).

2.3.3.1 Traditional risk factors

Extensive epidemiological research – starting with the Framingham study in 1948 – has contributed to the identification of various non-modifiable and modifiable risk factors, which are now termed as traditional risk factors for CAD (O'Donnell & Eosua, 2008) and account for most coronary events (Beaglehole & Magnus, 2002).

The best-established independent non-modifiable risk factors are *age*, *gender*, *ethnicity* (Nichols et al., 2012), and *genetics* (O'Donnell, 2004).
The risk of a CAD event increases with age and is higher in men than in woman (Mozaffarian et al., 2015). A 26-year follow-up of the Framingham population showed that among people aged 35 to 84 years, the total incidence of CAD morbidity and mortality is twofold higher in men than in woman. The sex difference is largest between the ages of 45 and 54 years, but tends to diminish during the later years of age (Lerner & Kannel, 1986). At the age of 40, the lifetime risk of developing CAD is 50% in men and 33% in women (Lloyd-Jones, Larson, Beiser & Levy, 1999; Roeters van Lennep, Westerveld, Erkelens & van der Wall, 2002). Although CAD is considered as a disease of ageing, genetic predispositions of CAD also contribute to the development of this disease (Scheuner, 2001). Several studies have shown a two-to-threefold higher risk for CAD in first-degree relatives. Furthermore, a positive family history of CAD approximately doubles the risk of CAD (Scheuner, 2001). Although the CAD risk in whites is at least as high as that in other racial / ethnic groups in the United States, death rates from CAD among African Americans are greater than those among Caucasians (Ferdinand, 2006).
Major modifiable risk factors – independent of age, gender, and ethnicity – include *smoking*, *diabetes mellitus*, *hyperlipidemia (high total serum cholesterol)*, and *hypertension* (Khot et al., 2003). Current smoking and hyperlipidemia are the two strongest risk factors, followed by diabetes mellitus and hypertension (Yusuf et al., 2004). Hyperlipidemia is determined by

unhealthy diet, physical inactivity, obesity, and their interaction (Magnus & Beaglehole, 2001). Importantly, modifiable risk factors account for 90% of the CAD risk (Yusuf et al., 2004), and their treatments have shown to reduce the risk of future cardiac events (Khot et al., 2003).

Although non-modifiable and modifiable traditional risk factors have shown to be independently associated with CAD (Ferdinand, 2006; Lerner & Kannel, 1986; Scheuner, 2001; Yusuf et al., 2004), they do not explain all of the risk for incident CAD events (Helfand et al., 2009). Therefore, the search for intermediate biological risk factors and psychosocial risk factors accounting for CAD causation is justified (Beaglehole & Magnus, 2002; Rozanski, Blumenthal, Davidson, Saab & Kubzansky, 2005).

2.3.3.2 Intermediate biological risk factors

To better predict future CAD, several intermediate biological risk factors such as blood lipids (also classified as traditional risk factors; Di Angelantonio et al., 2009; Sarwar et al., 2007), homocysteine (Castro, Rivera, Blom & Jakobs, 2006; Gauthier, Keevil & McBride, 2003), hemoconcentration (Allen & Patterson, 1995), coagulation parameters (e.g. fibrinogen; Danesh, Collins, Appleby & Peto, 1998), and inflammatory markers such as C-reactive protein (CRP; Kaptoge et al., 2010) and cytokines (Kaptoge et al., 2014) were found to be associated with an increased risk of CAD.

As outlined in section 2.3.1, inflammation plays a pivotal role in the initiation and progression of atherosclerosis (Hansson & Libby, 2006). For a better understanding of the inflammatory process in atherogenesis, inflammatory markers such as CRP and cytokines, which have been studied as potential predictors of CAD (Martins et al., 2006), will be presented in the following in more detail.

2.3.3.2.1 C-reactive protein

CRP, an acute-phase protein synthesized by the liver and regulated by proinflammatory cytokines (e.g. IL-6 and TNF-α), is known to be a sensitive marker of inflammation and tissue damage (Gauldie, Richards, Northemann, Fey & Baumann, 1989; Pepys & Hirschfield, 2003). CRP is particularly interesting in regard to cardiovascular biology and pathology for a variety of reasons: First, CRP binds to modified LDL, as found in atheromatous plaques;

second, CRP is deposited in the majority of plaques; and third, CRP has a range of proinflammatory properties that may contribute to the pathogenesis and progression of CAD (Hirschfield & Pepys, 2003). Hence, CRP is one of the most extensively studied inflammatory markers of CAD risk (Beaglehole & Magnus, 2002) and the only one recommended for clinical application (Martins et al., 2006).

The first population-based prospective study on the relation between CRP and cardiovascular outcomes was conducted in 1996 and reported an association between baseline CRP levels and CAD death in high-risk but healthy men (Kuller, Russelt, Tracy, Shaten & Meilahn, 1996). Since then, several meta-analyses have been published. They conclusively reported that CRP levels were independently associated with CAD events. However, the predictive value of CRP in CAD was insufficient (Buckley, Fu, Freeman, Rogers & Helfand, 2009; Danesh et al., 2004; Kaptoge et al., 2010). Therefore, a causal role of CRP in CAD remains to be elucidated (Strang & Schunkert, 2014).

2.3.3.2.2 Cytokines

Cytokines are intercellular signaling peptides (Cannon, 2000) and are involved in the process of inflammation, where they are classified into proinflammatory and anti-inflammatory cytokines (Dinarello, 2000). Proinflammatory cytokines (e.g. TNF-α) promote inflammation, whereas anti-inflammatory cytokines (e.g. IL-10) suppress the activity of proinflammatory cytokines (Dinarello, 2000). Proinflammatory cytokines are more likely to be aetiologically relevant to CAD (Kaptoge et al., 2014) because enhanced levels of proinflammatory cytokines, in particular TNF-α, may induce the production of ROS, resulting in endothelial dysfunction (Zhang et al., 2009), which is described as an initial step in atherosclerosis (Ross, 1999).

The most studied cytokine involved in inflammation is IL-6 (Kaptoge et al., 2014). A prospective study including apparently healthy men indicated that baseline levels of IL-6 are significantly associated with increased risk of future MI (Ridker, Rifai, Meir, Stampfer, Charles & Hennekens. 2000). Furthermore, a systematic review of prospective, population-based studies reported that long-term average IL-6 levels are positively associated with CAD risk. This association was as high as for some major traditional risk factors (Danesh et al., 2008).

There is less epidemiological evidence for the proinflammatory cytokine TNF-α (Kaptoge et al., 2014). However, TNF-α has been shown to be a significant independent predictor of

incident CAD and CVD events as well as total mortality among men (Tuomisto, Jousilahti, Sundvall, Pajunen & Salomaa, 2006), and is persistently elevated in MI survivors at increased risk for a recurrent event (Ridker, Rifai, Pfeffer, Sacks, Lepage & Braunwald, 2000). In a recent meta-analysis, higher baseline level (1 standard deviation) of either IL-6 or TNF-α was associated with an approximately 10-25% higher risk of non-fatal MI or of CAD death (Kaptoge et al., 2014).

It should be emphasized that although the results of Kaptoge and colleagues indicated that circulating levels of proinflammatory cytokines, including IL-6 and TNF-α, in initially healthy individuals were associated with risk of CAD outcomes, further studies are needed to assess causality (Kaptoge et al., 2014).

2.3.3.3 Psychosocial risk factors

As early as 1936, Menninger and Menninger suggested that psychological factors play an important role in the development of cardiac pathology. Since then, studies of psychosocial risk factors for CAD have provided convincing evidence that psychosocial factors contribute to the pathogenesis and adverse outcome of CAD (Rozanski, 2014). Notably, these psychosocial risk factors often cluster together in CAD patients and subsequently elevate the risk for cardiac events even more (Kupper & Denollet, 2007).

To date, various psychosocial factors have been reported to promote the development and progression of CAD (Rozanski et al., 2005; Rozanski, 2014). These risk factors are classified into three groups, based on their duration and temporal proximity to the occurrence of coronary syndromes: (1) chronic psychosocial risk factors, (2) episodic psychosocial risk factors, and (3) acute psychosocial risk factors (Kopp, 1999). Accordingly, the following section briefly describes the most important ones in each group and outlines their associations with CAD and / or CAD outcomes.

2.3.3.3.1 Chronic psychosocial risk factors

Chronic psychosocial risk factors promote gradual progression of CAD through an increased SNS activity and an adverse cardiovascular risk profile such as hypertension (Kopp, 1999).

Anger and hostility

Anger is usually defined as an emotional state that consists of feelings ranging in intensity from mild irritation or annoyance to intense fury or outrage, whereas hostility is characterized as a negative attitude directed toward other persons (Martin, Watson & Wan, 2000). Although there are important differences between these constructs, both terms are often used synonymously (Chida & Steptoe, 2009).

A meta-analysis including 25 studies in initially healthy populations and 19 studies in CAD patients reported that anger and hostility were significantly associated with increased CAD events in initially healthy populations and with a poor prognosis in CAD patients. In addition, the harmful effects of anger and hostility were greater in CAD patients than in healthy populations and greater in men than in women. However, as the harmful effects of anger and hostility on CAD lost significance in both healthy and CAD populations after controlling for the full spectrum of behavioral covariates (e.g. smoking, physical activity, BMI), the causal role of anger and hostility still remains an open question (Chida & Steptoe, 2009).

Job strain

Job strain – the psychosocial stress construct that is most frequently studied as a potential cause of CAD – is characterized as the combination of high job demands and low control at work (Kivimäki et al., 2012).

A recent meta-analysis involving 13 published and unpublished European cohort studies found a small but consistent association between job strain and increased risk of an incident event of CAD. This association was independent of lifestyle and traditional risk factors (Kivimäki et al., 2012). In addition, the risk of CAD has been shown to be highest among individuals with job strain who practice an unhealthy lifestyle. Individuals reporting job strain and a healthy lifestyle had about half the rate of disease, indicating that a healthy lifestyle may reduce CAD risk among individuals with job strain (Kivimäki et al., 2013). These studies were based on observational data. Thus, conclusions about causality are not possible because residual or unmeasured confounding factors may represent an alternative explanation for these results (Kivimäki et al., 2012; Kivimäki et al., 2013).

Low social support

Social support can be structured into two main domains: (1) structural social support (SSS), and (2) functional social support (FSS; Lett, Blumenthal, Babyak, Strauman, Robins &

Sherwood, 2005). SSS refers to the size, type, density, and frequency of contact with the surrounding network, whereas FSS refers to the support provided by the social structure, including instrumental, financial, informational, appraisal, and emotional support. Furthermore, FSS can be distinguished into received (i.e. is actually available) and perceived (i.e. is available if needed) functional support (Lett et al., 2005).

Generally, various types of low social support have been shown to be associated with an approximately 1.5-fold increased CAD risk both in healthy populations and in CAD patients (Lett et al, 2005). A recent meta-analysis including 5 etiological studies in apparently healthy populations and 20 prognostic studies with CAD patients reported an association between low FSS and the prevalence of CAD in healthy populations (Barth, Schneider & von Känel, 2010). In addition, FSS negatively affects cardiac and all-cause mortality in CAD patients. These results were not found for low SSS, suggesting that FSS is more important for CAD development and prognosis (Barth et al., 2010).

Anxiety

Anxiety is characterized as an emotional state involving feelings of apprehension, tension, nervousness, and worry accompanied by physiological arousal (Spielberger, 2010), and can manifest in different clinical disorders such as generalized anxiety disorder, panic disorder, and posttraumatic stress disorder (Olafiranye, Jean-Louis, Zizi, Nunes & Vincent, 2011).

Until recently, due to inconsistent results, the role of anxiety as a CAD risk factor was unclear (Rozanski, 2014). However, several recent meta-analyses have shed light on this question, by indicating that anxiety is an independent risk factor for incident CAD and cardiac mortality in initially healthy individuals (Roest, Martens, de Jonge & Denollet, 2010) as well as a prognostic risk factor in patients with MI (Roest, Martens, Denollet & de Jonge, 2010). Furthermore, it has been shown that individuals diagnosed with posttraumatic stress disorder (Edmondson, Kronish, Shaffer, Falzon & Burg, 2013) or panic disorder (Gomez-Caminero, Blutmentals, Russo, Brown & Castilla-Puentes, 2005; Walters, Rait, Petersen, Williams & Nazareth, 2008) are at increased risk of CAD as compared to individuals without these disorders. In addition, generalized anxiety disorder in CAD patients was independently associated with an increased risk of adverse outcomes (Martens, de Jonge, Na, Cohen, Lett & Whooley, 2010; Roest, Zuidersma & de Jonge, 2012).

2.3.3.3.2 Episodic psychosocial risk factors

Episodic psychosocial risk factors have a duration lasting from several months to two years, and tend to recur. Depression and vital exhaustion are considered important episodic psychosocial risk factors (Kopp, 1999).

Depression

Depressive disorders range from mild / subclinical depressive symptoms to major depression as a classical clinical manifestation. Major depression is mainly characterized by the presence of depressed mood and / or anhedonia (i.e. the inability to take pleasure in life) persisting for at least two weeks or more and is further accompanied by functional impairment and somatic complaints (Rozanski et al., 2005).

Over the past decades, most studies have focused on the role of depression in CAD (Roest et al., 2012) and have demonstrated that depression is a potent risk factor for CAD (Rozanski, 2014). Various meta-analyses reported that depression was independently associated with the development of CAD in the general population (Nicholson, Kuper & Hemingway, 2006; Van der Kooy, van Hout, Marwijk, Marten, Stehouwer & Beekman, 2007; Wulsin & Singal, 2003) and with an increased risk of adverse cardiovascular outcome and cardiac mortality or all-cause mortality in CAD patients (Barth, Schumacher & Herrmann-Lingen, 2004; Meijer et al., 2013; Meijer, Conradi, Bos, Thombs, van Melle & de Jonge, 2011; Nicholson et al., 2006). Notably, individuals with a first-ever depression after an MI seem to be at higher risk for future incidents (de Jonge, van der Brink, Spijkeman & Ormel, 2006).

Vital exhaustion

Vital exhaustion (VE) has been conceptualized as a state of unusual fatigue, loss of energy, increased irritability, and feeling of demoralization (Schuitemaker, Dinant, van der Pol & Appels, 2004).

Appels and Mulder conducted the first prospective study on the association between VE and MI in 3877 healthy civil servants during a 4.2-year follow-up period, and reported that VE is an independent short-term predictor of MI (Appels & Mulder, 1988). In this study, subjects who were exhausted at baseline had a twofold higher risk of MI. Importantly, this predictive value was most evident in the first year of follow-up (Appels & Mulder, 1988). Since then, various studies have replicated this association, showing, among others, that VE approximately triples the risk of MI after controlling for potential confounders in the general

population (Schuitemaker et al., 2004) and predicts the risk of CAD mortality in men (Cole, Kawachi, Sesso, Paffenbarger & Lee, 1999). Furthermore, a recent prospective study indicated that VE independently predicts long-term risk for incident adverse cardiac events (Williams, Mosley, Kop, Couper, Welch & Rosamond, 2010).

VE might also contribute to a worse cardiac prognosis. Studies have shown that VE is independently related to recurrent events after a successful coronary angioplasty (Kop, Appels, Mendes de Leon, de Swart & Bär, 1994; Mendes de Leon, Kop, de Swart, Bär & Appels, 1996), a nonsurgical procedure to remove coronary stenosis and related anginal symptoms (Kop, 1999).

2.3.3.3.3 Acute psychosocial risk factors

Acute psychosocial risk factors act as triggers for cardiac events within the first two hours after the risk factor occurs by activating sudden pathophysiological responses (Kop, 1999; Steptoe & Brydon, 2009). Acute psychosocial risk factors involve acute anger, acute mental stress (e.g. acute work stressors), acute depressed mood, as well as earthquakes, terrorist incidents, or sporting events (Steptoe & Brydon, 2009). It should be emphasized that there are methodological limitations in measuring acute psychosocial risk factors for cardiac events prospectively, because acute MI or sudden cardiac death cannot be anticipated in advance. Therefore, studies of acute psychosocial triggers are typically retrospective in nature, including population-based studies and patient interviews (Steptoe & Bydon, 2009).

Studies in CAD patients have shown that both acute anger and acute depressed mood in the two hours before onset of cardiac symptoms are independently associated with an increased CAD risk. The odds ratio for CAD after acute anger was 2.06, with a relative risk of 7.3 (Strike, Perkins-Porras, Whitehead, McEwan & Steptoe, 2006), whereas the odds ratio for CAD after acute depressed mood was 2.5, with a relative risk of 4.33 (Steptoe, Strike, Perkins-Porras, McEwan & Whitehead, 2006). Furthermore, the Swedish Stockholm Heart Epidemiology Program Study found that acute work stressors such as having high-pressure deadlines in the previous 24 hours were associated with a six-fold increased CAD risk compared with the period 24 to 48 hours before the CAD event (Möller, Theorell, de Faire, Ahlbom & Hallqvist, 2005).

Studies on both earthquakes (e.g. Northridge, Hanshin-Awaji) and terrorist incidents (e.g. 9/11) reported a marked increase in hospital admission for CAD up to two months after the

devastating and stressful experiences (Feng, Lenihan, Johnson, Karri & Reddy, 2006; Leor & Kloner, 1996; Suzuki et al., 1997).
Sporting events can also be stressful for supporters (Steptoe & Brydon, 2009). Analyses of cardiac events during the 2006 soccer World Cup in Germany indicated that the incidence of cardiovascular events in men during the days of matches involving the German team was 3.26 times higher than in the control period before and after the World Cup. The highest average incidence of events was found during the first two hours after the beginning of each match (Wilbert-Lampen et al., 2008).

Notably, although psychological interventions seem to reduce all-cause mortality or nonfatal MI as well as depression and anxiety in CAD patients (Gulliksson, Burell, Vessby, Lundin, Toss & Svärdsudd, 2011; Welton, Caldwell, Adamopoulos & Vedhara, 2009; Whalley et al., 2011), evidence of a definite beneficial effect on cardiac endpoints remains inconclusive (Perk et al., 2012).

2.3.4 NADPH oxidase-derived superoxide anion production in atherosclerosis and coronary artery disease

To shed more light on potential mediating biological mechanisms of cardiovascular risk in CAD, a growing body of animal and human studies has analyzed NADPH oxidase and NADPH oxidase-derived superoxide anion production from both monocytes / macrophages and vascular wall cells (i.e. VSMCs) in atherosclerosis as the underlying process of CAD (Libby, 2002; Ross, 1999).

2.3.4.1 Animal studies

NADPH oxidase-derived superoxide anion production of vascular and phagocytic cells may play an important role in the formation of atherosclerotic plaques and thus in the progression of atherosclerosis. This is evidenced by findings indicating that NADPH oxidase-derived superoxide anion production is increased in various animal models of atherosclerosis and that an impaired NADPH oxidase reduces superoxide anion production and atherosclerotic plaque formation in these models. In vascular segments of the aorta of a rabbit model of early atherosclerosis (i.e. Watanabe rabbits; hypercholesterolemia secondary to an LDL-receptor

defect; Warnholtz et al., 1999) and a rabbit model of chronic atherosclerosis (Watanabe heritable hyperlipidemic rabbits (WHHR); i.e. a chronic model of atherosclerosis; Miller, Gutterman, Rios, Heistad & Davidson, 1998), NADPH oxidase-derived superoxide anion production was up to threefold higher than in controls. In addition, in Watanabe rabbits (i.e. model of early atherosclerosis), the reduction of *vascular* NADPH oxidase-derived superoxide anion production, caused by a treatment with Ang II type 1 receptor blocker, was associated with a reduced atherosclerotic plaque formation and macrophage infiltration and consequently with an improvement of endothelial dysfunction (Warnholtz et al., 1999). A possible equal contribution to atherosclerotic lesion formation from NADPH oxidase-derived superoxide anion production of VSMCs and monocyte / macrophages was supported by a study by Vendrov and colleagues (2007). In this study, apolipoprotein E-deficient ($ApoE^{-/-}$) mice (i.e. a mouse model of human atherosclerosis) with nonfunctional NADPH oxidase in monocyte / macrophages (BMO) or nonfunctional NADPH oxidase in VSMCs (VWO) showed a decreased NADPH oxidase-derived superoxide anion production and total aortic atherosclerosis lesion area compared to controls. Moreover, aortic sections of BMO and VWO mice had smaller lesions with fewer macrophages compared to controls. In addition, BMO mice had significantly lower oxidized LDL plasma levels compared to control and VWO mice, whereas aortic sections of VWO mice showed decreased expression of cellular adhesion molecules compared to controls and BMO mice (Vendrov et al., 2007). These results indicate that NADPH oxidase of vascular and phagocytic cells affect atherogenesis by two distinct mechanisms: First, NADPH oxidase-derived superoxide anion production from VSMCs, and thus *vascular* NADPH oxidase-derived superoxide anion production, increases the expression of cell adhesion molecules and may subsequently enhance the infiltration of monocyte / macrophages into the lesion. Second, NADPH oxidase-derived superoxide anion production from monocyte / macrophages and thus *phagocytic* NADPH oxidase-derived superoxide anion production, induces LDL oxidation, and may therefore enhance the recruitment of monocytes into atherosclerotic lesions (Vendrov et al., 2007).

The potential contribution of the NADPH oxidase subunits $p47^{phox}$ and $gp91^{phox}$ to atherosclerotic lesion formation was studied in $p47^{phox}$ and $gp91^{phox}$ knockout $ApoE^{-/-}$ mice (Barry-Lane et al., 2001; Hsich et al., 2000; Judkins et al., 2010). However, the results are not conclusive. Whereas one study found a decreased NADPH oxidase-derived superoxide anion production from VSMCs and reduced atherosclerotic lesion sizes in $p47^{phox}$ knockout $ApoE^{-/-}$ compared to controls (Barry-Lane et al., 2001), another study did not (Hsich et al., 2000).

Similar results were found for gp91^{phox} knockout ApoE$^{-/-}$ mice. In a study by Barry-Lane and colleagues, gp91^{phox} knockout ApoE$^{-/-}$ mice showed no decrease in NADPH oxidase-derived superoxide anion production from VSMCs and no reduction in atherosclerotic lesion sizes (Barry-Lane et al., 2001). In contrast, a recent study found a reduced aortic NADPH oxidase-derived superoxide anion production, an enhanced NO bioavailability, and a significant reduction in atherosclerotic plaque formation along the length of the aorta from arch to iliac bifurcation in gp91^{phox} knockout ApoE$^{-/-}$ mice compared to controls (Judkins et al., 2010). Furthermore, compared to controls, ApoE$^{-/-}$ mice showed an upregulation of gp91^{phox} expression in aortic endothelial cells as well as in macrophages before the appearance of atherosclerotic lesions. This upregulation was associated with an elevated aortic NADPH oxidase-derived superoxide anion production. These results suggest that the expression of gp91^{phox} particularly in macrophages may have an additional role in the formation of oxidized LDL, as a major cause of atherosclerotic lesion formation (Judkins et al., 2010).

It should be stressed that it is difficult to induce coronary lesions in animal disease models of human atherosclerosis and related CAD. Thus, CAD progression and pathophysiological changes seen in these models are not representative of those seen in humans (Liao, Huang & Liu, 2015).

2.3.4.2 Human studies

Vascular NADPH oxidase-derived superoxide anion production may play an important role in the mediation of endothelial dysfunction and thus in the pathophysiology of human atherosclerosis. This is supported by studies indicating that an increased *vascular* NADPH oxidase-derived superoxide anion production is positively associated with endothelial dysfunction (Guzik et al., 2000a; Heitzer et al., 2001) as an initial step in atherosclerosis (Ross, 1999). In saphenous veins of CAD patients, increased superoxide anion production by *vascular* NADPH oxidase activation was associated with a reduced NO-mediated vasorelaxation. Furthermore, increased NADPH oxidase activity and reduced NO-mediated vasorelaxation were both associated with increased clinical risk factors for atherosclerosis, of which diabetes and hypercholesterolemia were independently associated with an increased NADPH oxidase-derived superoxide anion production (Guzik et al., 2000a). Moreover, in CAD patients, it has been shown that peripheral vascular endothelial dysfunction (by measuring forearm blood flow responses in the brachial artery to acetylcholine and sodium

nitroprusside) and *vascular* superoxide anion production predict the risk of cardiovascular events (Heitzer et al., 2001). However, in this study, superoxide anion production was assessed by measuring forearm blood flow responses to acetylcholine in response to a coinfusion of the antioxidant vitamin C. This method is based on previous findings indicating that vitamin C may act as a scavenger of superoxide anions and thus improve endothelial dysfunction in patients with CAD (Levine, Frei, Koulouris, Gerhard, Keaney & Vita, 1996) or patients with coronary risk factors (Heitzer, Just & Münzel, 1996; Ting, Timimi, Haley, Roddy, Ganz & Creager, 1997). Hence, neither superoxide anion production nor the exact source of superoxide anions (i.e. NADPH oxidase) was assessed. Therefore, these prospective results should be interpreted with caution (Heitzer et al., 2001).

Macrophages accumulating at the border of atheromatous plaques (i.e. the "shoulder region"), which is the most frequent site of plaque rupture, seem to be a major source of increased NADPH oxidase-derived superoxide anion production likely involved in the genesis and progression of human coronary atherosclerotic disease. This is supported by studies comparing nonatherosclerotic and atherosclerotic coronary arteries from CAD patients. These studies consistently demonstrate that the expression of $p22^{phox}$ and $gp91^{phox}$ NADPH oxidase subunits in atherosclerotic arteries was increased through the vessel wall (i.e. adventitia, neointima, media, and endothelium), with the highest expression in the macrophage-rich central region of the plaque shoulder (Azumi et al., 1999; Guzik et al., 2006; Sorescu et al., 2002), where superoxide anion production was found to be most intense (Sorescu et al., 2002). Moreover, the expression of both $p22^{phox}$ and $gp91^{phox}$ increased with increasing severity of atherosclerosis (Sorescu et al., 2002). This increased expression of $p22^{phox}$ and $gp91^{phox}$ was largely due to higher monocyte / macrophage infiltration (Guzik et al., 2006; Sourescu et al., 2002). Notably, an association between PMA-stimulated polymorphonuclear neutrophils and severity of atherosclerosis was not found, supporting the important role of other phagocytic superoxide anion-generating cells (e.g. macrophages) in the process of atherosclerosis (Wykretowicz et al., 2005).

NADPH oxidase-derived superoxide anion production from macrophages seems further to induce LDL oxidation as an important cause of endothelial dysfunction. This is evidenced by a study demonstrating that in atherosclerotic plaque obtained from directional coronary atherectomy (DCA, i.e. a method of coronary revascularization in which atherosclerotic plaque is removed) of CAD patients, the expression of $p22^{phox}$, oxidized LDL, and superoxide anion production is increased. Furthermore, superoxide anions, mainly generated from

accumulating macrophages, were significantly associated with the distribution of $p22^{phox}$ and oxidized LDL (Azumi et al., 2002). These findings may be supported by a previous study demonstrating that NADPH oxidase-derived superoxide anion production from activated human monocytes is required for oxidative modification of LDL (Bey & Cathcart, 2000).

Notably, a $p22^{phox}$ polymorphism has been identified which seems to protect against cardiovascular risk by reducing *vascular* NADPH oxidase-derived superoxide anion production. However, results are inconsistent. A study in CAD patients investigating the functional effect of the C242T polymorphism, which encodes $p22^{phox}$, on *vascular* NADPH oxidase-derived superoxide anion production showed that both saphenous veins and mammary arteries with C242T polymorphism were significantly associated with lower basal and stimulated *vascular* NADPH oxidase-derived superoxide anion production compared to those without C242T polymorphism. This association was independent of atherosclerotic risk factors, including hypercholesterolemia, smoking, diabetes, and hypertension (Guzik et al., 2000b). Although this observation is in line with a previous study indicating a protective effect of the C242T polymorphism in the $p22^{phox}$ gene on coronary risk due to the lower frequency of the C242T polymorphism found in CAD patients compared to controls (Inoue, Kawashima, Kanazawa, Yamada, Akita & Yokoyama, 1998), other studies in CAD patients reported either a significant association between C242T polymorphism and the progression of coronary atherosclerosis (Cahilly, Ballantyne, Lim, Gotto & Marian, 2000) or no association (Zafari et al., 2004). Therefore, further studies are needed to demonstrate whether the C242T polymorphism in the $p22^{phox}$ gene influences atherogenesis and the progression of CAD.

2.4 Type D personality

Personality, as a psychological concept, is described as a complex organization of trait dispositions (Denollet, 2000). These traits reflect regularities and consistencies in the general affective level and behavior of individuals (Denollet, 2000; Gangestad & Snyder, 1985) and may have more explanatory and prognostic power in the prediction of CAD outcomes than other psychosocial risk factors such as depression (Denollet, 2000; Kupper & Denollet, 2007; Pedersen & Denollet, 2003).

The two cardiologists Friedman and Rosenman were the first to associate different behavioral characteristics with CAD. In 1959 and 1961, they observed that men and woman with a specific overt behavior pattern had higher cholesterol levels, a more rapid clotting time, and a greater incidence of clinical CAD than those with a converse behavior pattern. These results were independent of calorie or fat intake, alcohol consumption, or smoking status (Friedman & Rosenman, 1959; Rosenman & Friedman, 1961). Based on these clinical observations, they characterized 'Type A behavior pattern' (TABP), also known as 'Type A personality', by a heightened degree of ambitiousness and competitiveness, aggressiveness and hostility, and impatience associated with an incessant struggle to achieve more in less time, and suggested that TABP was an important risk factor for the development of CAD (Friedman & Rosenman, 1974). In addition, they labeled 'Type B behavior pattern' (TBBP; also termed 'Type B personality') as the converse of TABP. TBBP is defined by the absence of Type A characteristics and is known as a non-coronary-prone behavior pattern (Friedman & Rosenman, 1974). Individuals with a TBBP are typically more relaxed, easy-going, satisfied, and unhurried (Rose, 1987), and therefore at lower risk for CAD (Friedman & Rosenman, 1974; Rose, 1987).

The hypothesis that TABP might be a risk factor in the pathogenesis of CAD was strongly supported by findings from the Western Collaborative Group Study (Jenkins, Rosenman & Zyzanski, 1974; Rosenman, Brand, Jenkins, Friedman, Straus & Wurm, 1975; Rosenman, Friedman, Straus, Jenkins, Zyzanski & Wurm, 1970) and the Framingham Study (Haynes, Feinleib & Kannel, 1980). These studies reported that TABP was significantly associated with an increased CAD incidence. Based on this convincing evidence, in 1981, a Review Panel of biomedical and behavioral scientists accepted TABP as a risk factor for CAD (Cooper, Detre & Weiss, 1981).

Nevertheless, subsequent studies failed to show a relationship between TABP and CAD (Barefoot et al., 1989; Bunker et al., 2003; Kuper, Marmot & Hemingway, 2002). Moreover, a re-analysis of the Western Collaborative Study showed that CAD survivors with TABP were at lower risk for CAD mortality compared to those with TBBP, suggesting that TABP is a protective factor (Ragland & Brand, 1988). These discrepancies – probably due to methodological problems in assessing TABP (i.e. interview or self-report methods) – led to doubts that global TABP is a risk factor for CAD (Lachar, 1993; Matthews & Haynes, 1986). Therefore, later studies focused on the multidimensional nature of TABP and indicated that separate subcomponents of TABP may contribute differentially (positively or negatively) to the pathogenesis of CAD (Lachar, 1993). Among these, anger / hostility has been shown to be the most toxic TABP subcomponent associated with CAD (Dembroski & Costa, 1987; Dembroski, MacDougall, Williams, Haney & Blumenthal, 1985; see section 2.3.3.3.1), even if global TABP failed to be predictive (Dembroski et al., 1985; Lachar, 1993).

Although TABP was particularly designed to avoid any associations with global personality traits (Dimsdale, 1988; Pedersen & Denollet, 2003), the inconclusive results concerning TABP and CAD (Lacher, 1993) led to a decline in interest in personality traits in cardiovascular research (Kupper & Denollet, 2007). However, over the past 20 years, there has been a revival of interest in the role of personality traits in health and disease (Denollet, 2000; Kupper & Denollet, 2007; Sanderman & Ranchor, 1997; Scheier & Bridges, 1995). In the course of this revival, a novel personality type, termed as Type D personality (Type D), has been proposed (Denollet et al., 1995).

2.4.1 Construct of Type D personality

In 1995, Denollet and colleagues first introduced the concept of Type D, or “distressed” personality. Notably, the term “distressed” refers to a discrete personality configuration characterizing individuals who are inclined to experience emotional and interpersonal difficulties that are likely to affect physical health (Denollet et al., 1995; Denollet, 2000). This non-psychopathological construct was originally developed in Belgian patients with CAD to investigate the role of personality traits in CAD outcome (Denollet, 1998; Pedersen & Denollet, 2003).

The conceptualization of Type D was derived from existing personality theory (i.e. the five-factor model of personality and social-emotional adjustment; McCrae & Costa, 1987; Weinberger & Schwarz, 1990) and empirical research using statistical cluster analysis (Denollet, 2000; Denollet et al., 1995; Pederson & Denollet, 2003). These studies in CAD patients demonstrated that Type D was characterized by the combination of high negative affectivity (NA) and high social inhibition (SI; Denollet & De Potter, 1992; Denollet, 1993), both familiar constructs in personality research (Kupper & Denollet, 2007). The reliability of this personality type was shown across parallel data sets and follow-up assessments by demonstrating that CAD patients with Type D experienced emotional distress 15 months after the first assessment (Denollet & De Potter, 1992; Denollet, 2000; Denollet et al., 1995). The operational definition of Type D results from a median split of total scores on self-report measures of NA and SI (Denollet, 2000; Denollet et al., 1996). Hence, individuals with Type D tend to simultaneously experience negative emotions such as irritability and worry (i.e. NA) and to inhibit self-expression in a social interaction (i.e. SI; Denollet, 2000; Denollet et al., 1996).

2.4.1.1 Negative affectivity

NA is a stable and broad personality trait that is characterized by the tendency to experience negative emotional states across time and situations (Kupper & Denollet, 2007; Watson & Clark, 1984). Individuals scoring high on NA experience more feelings of dysphoria and tension, have a negative view of themselves, report somatic symptoms, and have an attentional bias towards adverse stimuli (Denollet, 2000; Kupper & Denollet, 2007; Watson & Pennebaker, 1989). Because NA correlates highly (r = .68) with the neuroticism scale from the NEO-Five Factor Inventory (NEO-FFI) in healthy participants (de Fruyt & Denollet, 2002; Denollet, 2000; Kupper & Denollet, 2007) as well as with the neuroticism scale (r = .64) from the Eysenck Personality Questionnaire (EPQ) in CAD patients (Denollet, 1998; Denollet, 2000; Kupper & Denollet, 2007), NA has also been conceptualized as neuroticism (Denollet, 1991; Eysenck, 1991; McCrae & Costa, 1987), a personality trait consisting of chronic negative emotions and associated cognitive and behavioral characteristics such as preoccupation and insecurity (Denollet, 1991). Nevertheless, the shared variance of NA and neuroticism only ranges from 40% to 50%, suggesting that these constructs are closely related

but not identical (Denollet, 2000; Denollet, 2005), even though they are often used synonymously (Denollet, 2000; Suls & Martin, 2005).

Studies on NA indicated that individuals high in NA have a greater exposure and a stronger reactivity to stressful events (Bolger & Zuckerman, 1995; Suls & Martin, 2005), of which interpersonal stressors are the most frequent (Gunthert, Cohen & Armeli, 1999). Furthermore, NA is negatively associated with overall quality of life in healthy individuals (Arrindell, Heesink & Feij, 1999; Lynn & Steel, 2006; Ozer & Benet-Martinez, 2006) and CAD patients (Denollet, 1991). In addition, there is growing evidence that NA is associated with mental and physical health problems such as anxiety, depression (Lahey, 2009), and CAD (Suls & Bunde, 2005) and further predicts morbidity and mortality in individuals with chronic disease (e.g. type I diabetes) and cancer (Lahey, 2009).

2.4.1.2 Social inhibition

SI is a broad and stable personality trait representing the tendency to inhibit the expression of emotions, thoughts, and behaviors in social interaction (Asendorpf, 1993; Denollet, 1997; Kupper & Denollet, 2007) and has further been related to the avoidance of potential dangers involved in social interaction situations (Asendorpf, 1993; Denollet, 2000). Individuals high in SI perceive the outside world as threatening by anticipating negative reactions from others (e.g. disapproval, nonreward). As a consequence, they adopt self-enhancing strategies, including withdrawal (Denollet, 1997; Denollet, 2000) and excessive control over self-expression (Friedman & Booth-Kewley, 1987; Denollet, 2000). Therefore, high SI individuals may feel inhibited, tense, and insecure in the company of others (Asendorpf, 1993; Denollet, 2000; Kupper & Denollet, 2007). Thus, SI refers to pervasive individual differences in reticence, withdrawal, and nonexpression associated with negative emotionality and personal distress (Denollet, 2000; Kupper & Denollet, 2007).

On the continuum of the intrapersonal dimensions extraversion-introversion, SI has been shown to be closely related to introversion (Denollet, 1997). Introverted individuals tend to be quiet and concentrate on their inner worlds, do not like excitement and are distant from other people except for intimate friends. Therefore, they can function without the need for high levels of external stimulation (Eysenck & Eysenck, 1975; Hills & Argyle, 2001). Studies have shown that SI is inversely correlated with extraversion in healthy individuals (assessed by means of the NEO-FFI, r = -.59) and CAD patients (assessed by means of the EPQ, r = -.65),

respectively (Denollet, 2005). However, the common variance of these two personality traits only ranges from 25% to 45%, implying that they are related but not identical. Similar to NA and neuroticism, these constructs are usually used synonymously (Denollet, 2000).

Studies have shown that SI is associated with low self-esteem (Denollet, 2000), a less positive and less active social life (Gest, 1997), and poor emotional well-being (Denollet, 2000) as well as increased cardiovascular reactivity (Cole, Kemeny, Weitzman, Schoen & Anton, 1999; Gross & Levenson, 1997), decreased heart rate variability (HRV; Carpeggiani et al., 2005), and increased inflammation (Denollet et al., 2006a). The latter three associations were discussed as possible pathways by which SI may influence poor cardiac prognosis in CAD patients (Denollet et al., 2006a). Furthermore, SI has been related to low social coping (i.e. seeking of social support; Eisenberg, Fabes & Murphy, 1995; Kupper & Denollet, 2007) and low perceived social support (Denollet, 2000). Low perceived social support, in turn, has been associated with CAD development and prognosis (Barth et al., 2010; Rozanksi et al, 2005; see section 2.3.3.3.1).

Notably, the way in which individuals cope with negative emotions (i.e. inhibition) seems to be as important as the experience of negative emotions per se (Denollet, 2000). Therefore, only the synergistic effect of NA and SI shows a particular adverse effect on health (Denollet et al., 2006a; Denollet, Pedersen, Vrints & Conraads, 2013; Kupper & Denollet, 2007; Pedersen & Denollet, 2003).

2.4.1.3 Type D personality and depression

After Type D was introduced (Denollet et al., 1995), some critics speculated that Type D is nothing more than depression (Lespérance & Frasure-Smith, 1996). However, Type D refers to more than just a measure of negative affect or depression, due to the inclusion of SI, and therefore emphasize on how individuals cope with this affect (Pedersen & Denollet, 2006). In addition, depression reflects an episodic psychiatric disorder while Type D refers to a normal, chronic disposition (Denollet, Schiffer & Spek, 2010; see Table 6). Notably, most individuals with Type D do not meet diagnostic criteria for depression (Denollet et al., 2009; Denollet et al., 2010) or self-reported depression (Denollet et al., 2010; Denollet & Pedersen, 2008), indicating that Type D and depression may only partly overlap (Denollet et al., 2010). Indeed, follow-up studies reported that Type D still predicts adverse clinical outcome in CAD patients

independently of symptoms (Denollet & Brutsaert, 1998; Denollet & Pedersen, 2008; Denollet et al., 2010; Denollet, Vaes & Brutsaert, 2000) and severity (Denollet et al., 2010; Martens, Mols, Burg & Denollet, 2010) of depression. Moreover, Type D may be predictive for the onset, prevalence, persistence, and severity of depression in CAD patients, independently of baseline depression scores (Denollet et al., 2010). Importantly, these results do not imply that Type D is a better predictor than depression (Denollet et al., 2010), but future research may benefit from the inclusion of Type D in addition to other well-known psychosocial risk factors (Kupper & Denollet, 2007).

Table 6

Main differences between Type D personality and depression. Pedersen & Denollet (2006).

Construct	**Negative Emotions**	**Social inhibition**	**Duration**
Depression	Depressed affect in particular	Not specified	Episodic (< 2 years)
Type D personality	Negative affect in general (including worry, irritability)	Elevated levels (non-expression)	Chronic (≥ 2 years)

2.4.1.4 Type D personality as a determinant of psychosocial distress

Psychological distress is a nonspecific term including unpleasant feelings and emotions such as sadness, frustration, anxiety, and a variety of other emotional and social difficulties (Carney & Freedland, 2002).

Evidence suggests that emotional stress, chronic tension, anger, a low level of subjective well-being as well as pessimism, depressive symptoms, general negative affect, and a lack of perceived social support are more prevalent in CAD patients with Type D compared to those without Type D (Denollet, 2000; Pedersen & Denollet, 2003). Furthermore, a study of 171 CAD patients indicated that Type D was independently associated with a six-fold increased risk of VE at baseline and an approximately five-fold risk at six-week follow-up after treatment (Pedersen & Middel, 2001). Thus, it is not surprising that CAD patients with Type D show a relative absence of positive emotions based on their low level of self-esteem and a general dissatisfaction with life (Pedersen & Denollet, 2003).

The findings reported above underline the validity of the Type D construct as a 'distressed' personality profile that is related to an increased vulnerability to emotional and social difficulties (Pedersen & Denollet, 2003).

2.4.2 Assessment of Type D personality

Over the past years, various methods of assessment have been used to identify Type D (Kupper & Denollet, 2007).

Initially, Type D was assessed by a combination scale method using the Social Inhibition Scale of the Heart Patients Psychological Questionnaire (HPPQ; Erdmann, Duivenwoorden, Verhage, Kazemier & Hugenholtz, 1986) for the assessment of SI and the Trait Scale of the State-Trait Anxiety Inventory (STAI; Van Der Ploeg, Defares & Spielberger, 1980) for the assessment of NA (Denollet et al., 1995; Denollet et al., 1996; Kupper & Denollet, 2007). Afterwards, a median split on these measures (i.e. trait-anxiety $\geq$ 43 and social inhibition $\geq$ 12) was used to classify Type D (Denollet et al., 1995; Denollet et al., 1998). Although these scales have been shown to be valid measures of both NA and SI in CAD patients (Denollet, 1991; Denollet & De Potter, 1992), the internal consistency of the Social Inhibition Scale from Erdmann and colleagues (1986) has proved to be rather poor. Therefore, the Type D scale-16 (DS16) was developed to fill the gap of measurement instruments and generate a standard for assessing NA, SI, and Type D. Thus, the DS16 provides an adequate characterization of individual risk when looking at the impact of Type D on CAD (Denollet, 2005; Kupper & Denollet, 2007).

In 1998, the DS16 was introduced as a brief self-report 16-item questionnaire for the assessment of Type D (Denollet, 1998). The items were selected from a pool of 66 statements derived from an item-level factor analysis of the Minnesota Multiphasic Personality Inventory (Johnson, Null, Butcher & Johnson, 1984) and self-constructed statements that were specifically written for the development of a Type D scale. Principal components analysis and internal consistency analysis were used to produce a short scale including 8 NA items (six items were related to dysphoria, whereas two items were related to anxiety and irritability) and 8 SI items (five items were related to discomfort and social poise, whereas three items were related to dominance). These 16 items were selected based on their ability to (1) discriminate between patients with and without Type D and (2) adequately reflect the

personality traits of Type D. In a first step, Type D was assessed using the combination scale method described above. Consistent with this method, all selected NA and SI items differentiated between patients with and without Type D. Furthermore, principal component analysis indicated that the selected items were clearly related to both NA (Cronbach's $\alpha = .89$) and SI (Cronbach's $\alpha = .82$). Test-retest reliability over a three-month interval was .78 for NA and .87 for SI. Moreover, the DS16 was validated against standard personality scales such as the Social Inhibition Scale of the HPPQ (Erdman et al., 1986) and the Trait Scale of the STAI (Van Der Ploeg et al., 1980). These results were replicated in a confirmatory analysis of an independent sample of CAD patients, suggesting that the DS16 is a reliable and valid instrument for the measurement of the personality traits NA and SI that define Type D (Denollet, 1998).

In 2000, the DS16 was revised to include the most prominent low-order traits related to the NA and SI personality dimensions (Denollet, 2000). Therefore, four new NA and six new SI items were added to better reflect the low-order trait of tension / worry and to enhance the assessment of the low-order trait of withdrawal, respectively. The three SI items related to dominance were deleted and replaced by a new SI item in order to adequately assess the low-order trait of reticence / nonexpression. This strategy resulted in a pool of 24 items comprising 12 NA items, reflecting the low-order traits of dysthymia and tension / worry with six items each, and 12 SI items reflecting the low-order traits of reticence / nonexpression and withdrawal with six items each. Statistical analyses indicate a high level of internal structural validity of the newly added NA and SI items (Denollet, 2000).

To devise a brief measure with little burden to patients, the DS16 was revised again into the Type D scale-14 (DS14; Denollet, 2005).

2.4.2.1 Type D scale-14

The DS14, as the most recent and shortest form of the Type D scales (Kupper & Denollet, 2007), was developed in 2005 by Denollet and specifically constructed to measure NA, SI, and thus Type D in a reliable and standardized way that poses lower respondent burdens.
The DS14 consists of 14 items (i.e. 7 items assessing NA and 7 items assessing SI; see Table 7) derived from its precursor DS16 (Denollet, 1998) and from the revised items, which were particularly generated to improve the assessment of NA and SI (Denollet, 2000). The item

selection was based on conceptual and psychometric reasons. Conceptually, the 7 NA items had to involve feelings of dysphoria (items 4, 7, and 13; see Table 7), anxious apprehension (items 2 and 12; see Table 7), and irritability (items 5 and 9; see Table 7), whereas the 7 SI items had to cover social discomfort (items 6, 8, and 14; see Table 7), reticence (items 10 and 11, see Table 7), and lack of social poise (items 1 and 3; see Table 7; Denollet, 2000; Denollet, 2005). Psychometrically, items with the highest item-total correlations were selected (Denollet, 2005). Factor analysis to examine the internal-structural validity of the NA and SI items revealed the presence of two dominant personality domains, with all 7 NA items and 7 SI items loading highly on their corresponding trait factor. Furthermore, it was shown that the 7 NA items of the NA scale covered dysphoria, worry, and irritability, whereas the 7 SI items of the SI scale covered discomfort in social interactions, reticence, and social poise. The NA and SI scales were internally consistent (Cronbach's α of 0.88 and 0.86 for NA and SI, respectively), stable over a 3-month period with a test-retest reliability of 0.72 and 0.82 for NA and SI, respectively, and independent of mood and health status. Moreover, NA was positively associated with neuroticism (r = 0.68) and SI was negatively associated with extraversion (r = - 0.59), confirming the construct validity of the DS14 against the NEO-FFI. In addition, Type D was associated with a four-fold increased risk of cardiovascular morbidity, independent of age and gender (Denollet, 2005). A recent cross-cultural analysis, including 6222 CAD patients from 21 different countries across the world, supported the two-factor structure and reliability of the DS14, indicating that the DS14 is a reliable and valid instrument for assessing NA, SI, and thus Type D (Kupper, Pedersen, Höfer, Saner, Oldrige & Denollet, 2013). Moreover, the DS14 is recommended for clinical practice as a screening tool in CAD patients (Albus, Jordan & Hermann-Lingen, 2004).

Table 7

Items of the DS14. Adapted from Denollet (2005).

DS14
1. *I make contact easily when I meet people*
2. I often make a fuss about unimportant things
3. *I often talk to strangers*
4. I often feel unhappy
5. I am often irritated
6. I often feel inhibited in social interactions
7. I take a gloomy view of things
8. I find it hard to start a conversation
9. I am often in a bad mood
10. I am a closed kind of person
11. I would rather keep other people at a distance
12. I often find myself worrying about something
13. I am often down in the dumps
14. When socializing, I don't find the right things to talk about

Note: Italic Items of the DS14 represent reverse-scored items.

Overall, the DS14 is a brief and easy-to-use self-administered questionnaire and its completion generally takes 5 to 10 minutes (Pedersen & Denollet, 2006). The 14 items are rated on a 5-point Likert scale (0 = "false", 1 = "rather false", 2 = "neutral", 3 = "rather true", 4 = "true"). The total scores for each subscale range between 0 and 28. Notably, the DS14 reveals four personality types (i.e. restrained = low NA / low SI, introverted = low NA / high SI, excitable = high NA / low SI, and Type D = high NA / high SI), but only those individuals who score highly on both NA and SI are classified as Type D (Kupper & Denollet, 2007). The presence of Type D is defined as having a cut-off score greater or equal to 10 on both subscales (Denollet, 2005). In addition to this Type D dichotomy, the interaction of continuous NA and SI scores can be assessed as a continuous single measure of Type D (Denollet et al., 2013).

The DS14 has been validated in multiple languages, making it widely applicable (Kupper & Denollet, 2007). For the purpose of this thesis the validated German version of the DS14 (Grande et al., 2004) was used. The psychometric properties of the German version were good with Cronbach's α of 0.87 and 0.86 for NA and SI, respectively. Further, the two-factor structure of the original instrument could be replicated and the construct validity was confirmed (Grande et al., 2004). The German version of the DS14 is represented in the Appendix (see A1 on p. 157).

2.4.3 Type D personality and cardiovascular health in patients with coronary artery disease

In 1996, Denollet and colleagues published the first study reporting an association between Type D and higher mortality in CAD patients. In this longitudinal study, 303 CAD patients were screened for Type D at baseline, and the amount of cardiac and non-cardiac deaths after a 6-10-year follow-up period were documented. Results indicated that Type D was a significant prognostic factor for cardiac and non-cardiac death, independent of the severity of cardiac disease (e.g. left ventricular function, extent of coronary obstructive disease), and of biomedical (e.g. age, history of myocardial infarction) and psychosocial (e.g. social alienation, depression) predictors. Since then, research on Type D in CAD patients has arisen (Grande, Romppel & Barth, 2012).

In a study of 87 CAD patients with a reduced left ventricular ejection fraction (LVEF), Type D was an independent predictor of long-term cardiac events (Denollet & Brutsaert, 1998). Furthermore, the co-occurrence of reduced LVEF, premature onset of CAD (≤ 55 years), and Type D increased the risk of adverse health outcome four-fold (Denollet et al., 2000). Findings of a further study examining the influences of stress and Type D on five-year prognosis in CAD patients indicated that both stress and Type D were associated with a nearly three-fold increased risk of an adverse cardiac event. Moreover, Type D predicted major adverse cardiac events (i.e. cardiac death, MI, and revascularization) even after adjusting for concurrent symptoms of stress and potential biomedical confounders. These results suggested that Type D reflects more than transient changes in stress, thus strengthening the notion that Type D is a stable personality type that significantly improves risk stratification (Denollet et al., 2006b).

Type D has also been shown to be an independent risk factor for poor prognosis and increased mortality not only in CAD patients (Denollet et al., 1996; Denollet et al., 2000; Denollet et al., 2006a) but also in patients with CHF (Schiffer, Smith, Pedersen, Widdershoven & Denollet, 2010) and peripheral arterial disease (i.e. a manifestation of generalized atherosclerosis; Aquarius, Smolderen, Hamming, De Vries, Vriens & Denollet, 2009) as well as in patients after heart transplantation (Denollet, Holmes, Vrints & Conraads, 2007) or implantation of an implantable cardioverter defibrillator (Pedersen, van den Broek, Erdman, Jordaens & Theuns, 2010).

Despite these promising findings, there were also several studies which reported no association between Type D and mortality in cardiac patients (de Voogd et al., 2009; Grande, Romppel, Vesper, Schubmann, Glaesmer & Hermann-Lingen, 2011; Pelle, Pedersen, Schiffer, Szabo, Widdershoven & Denollet, 2010; Volz, Schmid, Zwahlen, Kohls, Saner & Barth, 2011). Nevertheless, two recent meta-analyses including cross-sectional and prospective studies indicated that Type D is independently associated with a more than three-fold increased risk of morbidity and mortality in cardiac patients (Denollet et al., 2010; O'Dell, Masters, Spielmans & Maisto, 2011). An updated meta-analysis including more recent observational studies and studies with conflicting results supported the overall findings of an association between Type D and poor cardiac prognosis and increased mortality in cardiac patients. However, the strength of this association was lower than those published in previous meta-analyses. Furthermore, findings of this meta-analysis suggested that Type D affects prognosis only in CAD and not CHF patients (Grande et al., 2012).

Type D has also been related to psychological indicators of poor prognosis (O'Dell et al., 2011). Studies have shown that Type D independently predicts anxiety, poor mental health, VE (Denollet et al., 2010), and the onset (Pedersen, Ong, Sonnenschein, Serruys, Erdman & van Domburg, 2006) and persistence of depressive symptoms (Romppel, Herrmann-Lingen, Vesper & Grande, 2012) in CAD patients. In addition, meta-analyses indicated that Type D was associated with a two-fold increased risk for impaired physical health, a 2.5-fold increased risk for impaired mental health status (Versteeg, Spek, Pedersen & Denollet, 2012), and a poor health-related quality of life (O'Dell et al., 2011) not only in cardiac patients but also in the general population (Mols & Denollet, 2010). CAD patients with Type D also have more dysfunctional illness perception (e.g. they believe that their illness will be less controllable by themselves or through treatment; Williams, O'Connor, Grubb & O'Carroll,

2011a) and have poor adherence to medical treatment (Williams, O'Connor, Grubb & O'Carroll, 2011b) than CAD patients without Type D. Thus, general distress, dysfunctional illness perception, and poor medication adherence may represent potential mechanisms which help to explain the adverse cardiac outcomes in CAD patients with Type D.

2.4.3.1 Potential biological mechanisms underlying Type D personality and poor cardiac prognosis

The pathways underpinning the observed association between Type D and poor cardiac prognosis in CAD patients are not fully understood (Whitehead, Perkins-Porras, Strike, Magid & Steptoe, 2007). Nevertheless, several biological mechanisms have been suggested to play a role in this association (Denollet & Conraads, 2011; Kupper & Denollet, 2007).

2.4.3.1.1 Dysregulation of the hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal (HPA) axis is a central regulatory and control system, which plays an important role in the regulation of adaptive responses to stress. Cortisol – the endproduct of the axis released by the adrenal glands – is an important steroid hormone that regulates metabolic, cardiovascular, immune, and behavioral processes (Smith & Vale, 2006). Continued or repeated exposure to stress may lead to a chronic oversecretion of cortisol, with potentially harmful effects such as hypertension and cardiovascular disease (Kupper & Denollet, 2007).

Dysregulation of the HPA axis, resulting in elevated cortisol levels, has been discussed as a possible pathway in CAD patients with Type D (Sher, 2005). Studies have shown that Type D was associated with greater cortisol reactivity to acute stress in healthy individuals (Habra, Linden, Anderson & Weinberg, 2003). Furthermore, Type D was associated with higher awakening (Whitehead et al., 2007) and daytime (Molloy, Perkins-Porras, Strike & Steptoe, 2008) cortisol levels, independent of demographic (e.g. age, BMI) and clinical (e.g. history of hypertension and diabetes, number of diseased vessels) factors, as well as depression in CAD patients. These studies indicated that Type D is associated with a impairment of the HPA axis function (Habra et al., 2003; Molloy et al., 2008, Whitehead et al., 2007) and may contribute to biological (Molly et al., 2008) and inflammatory responses (Whitehead et al., 2007) influencing cardiac prognosis.

2.4.3.1.2 Dysregulation of the autonomic nervous system

The autonomic nervous system (ANS) is a regulatory system involved in the maintenance of the inner milieu of the body (i.e. homeostasis), including the gastrointestinal tract, the lower urinary tract, the genital tract, part of the airway, and the cardiovascular system. Basically, the most important ANS divisions are the sympathetic (SNS) and parasympathetic (PNS) nervous system. Whereas the SNS, among others, accelerates HR and raises BP, the PNS slows these two parameters and further increases gastrointestinal activity. Moreover, the ANS interacts with the endocrine system and the immune system (Gabella, 2012).

Dysregulation of the ANS has been proposed as a further potential underlying mechanism by which Type D affects cardiovascular disease development and prognosis (Kupper, Pelle & Denollet, 2013). Studies in healthy individuals have shown that in response to acute mental stress, Type D was associated with an increased SBP and DBP (Kupper et al., 2013), a decreased HRV (Martin et al., 2010), a higher resting HR (Einvik et al., 2011), but a reduced HR stress reactivity (Howard, Hughes & James, 2011). In cardiac patients with Type D, a reduced HR reactivity but unaltered BP reactivity in response to acute mental stress (Kupper, Denollet, Widdershoven & Kop, 2013) and a reduced HR recovery after exercise (von Känel et al., 2009) were reported. Excessive sympathetic or inadequate parasympathetic modulation of HR have been shown to predict cardiac prognosis (Lahiri, Kannankeril & Goldberger, 2008) by, among others, an upregulated proinflammatory cytokine production such as TNF-α and IL-6 (Tracey, 2007).

2.4.3.1.3 Dysregulation of the immune system

Dysregulation of the immune system has been discussed as a third and novel promising potential underlying mechanism that may explain the association between Type D and poor cardiac prognosis in CAD patients (Denollet & Conraads, 2011).

In healthy participants, Type D was associated with a higher concentration of CRP (Einvik et al., 2011). Furthermore, in CHF patients with and without LVEF, Type D was found to independently predict increased circulating levels of the proinflammatory cytokine TNF-α and TNF-α soluble receptors (Conraads et al., 2006; Denollet et al., 2003) but not IL-6 (Denollet, Vrints & Conraads, 2008).

Evidence suggests that TNF-α is an independent predictor of incident CAD, CVD events, and total mortality among men (Tuomistostu et al., 2006) and it has further been associated with

recurrent cardiac events following MI (Ridker et al., 2000; see section 2.3.3.2.2). Moreover, a study indicated that elevation in TNF-α receptor levels is associated with carotid atherosclerosis measured by means of maximal carotid plaque thickness (Elkind et al., 2002). TNF-α has been shown to be involved in atherogenesis and atherosclerosis by inducing the production of ROS, which leads to endothelial dysfunction (Zhang et al., 2009). Furthermore, TNF-α stimulates adhesion and migration of inflammatory cells into the arterial wall and activates macrophages. This process can cause migration of smooth muscle cells and subsequent plaque instability. In addition, TNF-α regulates the interaction between endothelium and blood platelets as well as clotting and fibrinolytic factors, resulting in enhanced platelet aggregation and subsequent thrombosis (Denollet et al., 2003). However, the findings of increased circulating levels of TNF-α and TNF-α soluble receptors in CHF patients with Type D (Conraads et al., 2006; Denollet et al., 2003) should be interpreted with caution, because these data were only adjusted for sex, age, ischemic etiology, and disease severity and not for metabolic variables known to influence on systemic inflammation. Thus, increased inflammation may be mediated by other risk factors (e.g. BMI) associated with Type D (Einvik et al., 2011).

In a more recent study in CHF patients, Type D was associated with increased oxidative stress in terms of higher serum levels of the oxidant marker xanthine oxidase together with lower serum levels of the antioxidant marker heat shock protein (Hsp) 70 (Kupper et al., 2009). Notably, oxidative stress is characterized by an imbalance between the production of ROS and antioxidant defense mechanisms (Betteridge, 2000). In addition, a very recent study reported an independent association between Type D and endothelial dysfunction, as indicated by the sum score of the plasma biomarkers soluble vascular cell adhesion molecule-1, soluble intercellular adhesion molecule-1, E-selectin, and Von Willebrand factor (van Dooren et al., 2016). Importantly, endothelial dysfunction, in which inflammatory processes are implicated, accelerates atherosclerosis and has been shown to be an independent predictor of poor cardiac prognosis in CAD patients (Halcox et al., 2002; Heitzer et al., 2001; Suwaidi et al., 2000).

Besides these three potential underlying mechanisms described above, Type D has also been related to traditional risk factors such as hypertension, diabetes, and a family history of CAD in healthy individuals (Svansdottir et al., 2013). Furthermore, an association between Type D and biological markers such as higher BMI, lower HDL, and higher serum triglyceride as well

as lifestyle-related cardiovascular risk factors including smoking and physical inactivity has been reported (Einvik et al., 2011; Svansdottir et al., 2013). In addition, an independent 12% higher calculated 10-year risk of developing CAD and a higher incidence of previous cardiac events was found in the general population with Type D (Svansdottir et al., 2013). These results indicate that unhealthy lifestyles may also partly explain the adverse outcome in individuals with Type D (Svansdottir et al., 2013).

2.4.4 NADPH oxidase-derived superoxide anion production in coronary artery disease patients with Type D personality

Although Type D has been shown to be associated with oxidative stress (Kupper et al., 2009) and endothelial dysfunction (van Dooren et al., 2016), both processes in which superoxide anions play an important role, the relation of Type D and superoxide anion production in CAD remains unclear. To date, and to the best of our knowledge, no study has examined the role of either *vascular* or *phagocytic* NADPH oxidase-derived superoxide anion production – as a further potential biological / immunological mechanism underlying Type D and poor cardiac prognosis – in CAD patients with Type D.

2.5 Summary of the theoretical background

Macrophages are tissue-based immune cells (i.e. white blood cells or leukocytes) which play a crucial role in both the innate and the adaptive immune system. There are two kinds of marcophages: (1) tissue-specific macrophages (e.g. osteoclasts, alveolar macrophages, microglial cells, and Kupffer cells) and (2) inflammatory macrophages. Tissue-specific macrophages are continually present in the tissue, whereas inflammatory macrophages are only present in the inflamed tissue under inflammatory conditions. To increase their competence for host defence during inflammation, inflammatory macrophages need to be activated by signals from their microinviroment. Depending on the type of activating signals, inflammatory macrophages can be polarized in functional phenotypes of these classically activated macrophages (M1) and alternatively activated macrophages (M2), representing the two extremes of a linear scale of macrophage phenotypes. Whereas M2 macrophages suppress inflammation, M1 macrophages induce inflammation. Furthermore, M1 macrophages display an enhanced microbicidal activity (i.e. the ability to kill microbes), which is primarily mediated by the production of ROS, particularly superoxide anions, generated by activated NADPH oxidase located in the phagolysosomal and plasma membrane of macrophages.

Essential hypertension is a chronic medical condition characterized by a sustained elevation of BP (SBP $\geq$ 140 mm Hg and / or DBP $\geq$ 90 mm Hg) in the arteries of unknown cause and is one of the primary risk factors for CVD, in particular CAD. CAD, in turn, is the most common type of heart disease and is considered the global leading public health burden. Atherosclerosis – a progressive inflammatory process of arterial wall thickening – is the underlying pathophysiological process of CAD, with inflammatory macrophages playing a pivotal role in both initiation and progression of atherosclerosis. Increasing evidence from animal and human studies of hypertension and CAD suggests that NADPH oxidase-derived superoxide anions are likely to play an important role in the pathogenesis of atherosclerosis. Indeed, increased NADPH oxidase-derived superoxide anion production has been identified in hypertension and CAD, supposed to promote atherosclerosis by reducing NO bioavailability and increasing the induction of LDL oxidation – both important causes of endothelial dysfunction – and further by facilitating monocyte / macrophage infiltration into the intima.

Type D, mostly assessed by the DS14, is defined as a tendency to experience negative emotions (i.e. NA), and to inhibit their expression in a social context (i.e. SI). This personality trait has been shown to be an independent psychosocial risk factor for poor cardiac prognosis and increased mortality in CAD patients. Although the pathway underlying the observed association between Type D and poor cardiac prognosis in CAD is not fully understood, several biological mechanisms have been suggested to explain this association: (1) dysregulation of the HPA axis resulting in sustained and increased cortisol levels, (2) dysregulation of the ANS resulting in increased SBP and DBP, decreased HRV, and higher HR, and (3) dysregulation of the immune system resulting in increased levels of TNF-α and TNF-α soluble receptors, increased oxidative stress, and endothelial dysfunction. To date, NADPH oxidase-derived superoxide anion production in CAD patients with Type D has not been studied.

3 AIMS AND HYPOTHESES OF THE EMPIRICAL STUDIES

Animal and human studies (see sections 2.2.5 and 2.3.4) indicate that NADPH oxidase activity and NADPH oxidase-derived superoxide anion production from both VSMCs and phagocytic cells are increased in essential hypertension (Fortuño et al., 2004; Maeda et al., 2003; Touyz & Schiffrin, 2001; Zalba et al., 2000) and CAD (Guzik et al., 2000a; Guzik et al., 2006; Judkins et al., 2010; Vendrov et al., 2007). These increases may contribute to the pathogenesis and progression of atherosclerosis by inducing endothelial dysfunction possibly via diminishing NO bioavailability (Fortuño et al., 2004; Judkins et al., 2010) and increasing the induction of LDL oxidation with subsequently enhanced recruitment of monocytes / macrophages into the atherosclerotic lesion (Vendrov et al., 2007).
Although NADPH oxidase-derived superoxide anion production from human monocyte-derived inflammatory M1 macrophages has not been investigated either in essential hypertension or in CAD, several studies indicate that NADPH oxidase-derived superoxide anions by phagocytic cells may play a pivotal role in essential hypertension and atherosclerosis as the underlying process of CAD (Fortuño et al., 2004; Watanabe et al., 2006).

In partially treated individuals with essential hypertension, an overproduction of NADPH oxidase-derived superoxide anion in PMA-stimulated PBMCs (i.e. monocytes and lymphocytes), as circulating macrophage precursor cells, was observed compared to normotensive controls (Fortuño et al., 2004). Furthermore, serum NO concentration was lower in hypertensive participants than in normotensives, suggesting that NADPH oxidase-derived superoxide anion by stimulated PBMCs might be involved in diminishing NO bioavailability, and may therefore contribute to endothelial dysfunction as an initial step in atherosclerosis (Fortuño et al., 2004). In addition, in untreated participants with essential hypertension compared to normotensives, ROS formation by PBMCs was positively associated with carotid artery IMT, as a vague index for atherosclerosis severity (Watanabe et al., 2006). In a very recent study, higher NADPH oxidase-derived superoxide anion production from PMA-stimulated PBMCs was found in treated hypertensive individuals with LVH compared to those without LVH and normotensive controls. These results suggest that

circulating macrophage precursor cells may contribute to hypertensive heart disease through an increased NADPH oxidase-derived superoxide anion production (Moreno et al., 2014).
In CAD patients, NADPH oxidase-derived superoxide anion production was found in nonatherosclerotic and atherosclerotic coronary arteries. However, in atherosclerotic arteries, an intense area of superoxide anion production by upregulated NADPH oxidase was found in the macrophage-rich plaque shoulder (i.e. the most frequent site of plaque rupture; Azumi et al., 1999; Sorescu et al., 2002). Moreover, in CAD patients, an increased activation of NADPH oxidase was found to be positively associated with severity of atherosclerosis due to increased macrophage infiltration, indicating that increased NADPH activity might be an important mechanism underlying increased NADPH oxidase-derived superoxide anion production in human CAD (Guzik et al., 2006; Sorescu et al., 2002). Additionally, in patients with angina pectoris, superoxide anion production by accumulating macrophages was significantly associated with the distribution of oxidized LDL, suggesting that macrophages are likely to induce LDL oxidation as an important cause of endothelial dysfunction, and thus play an important role in the pathogenesis of CAD (Azumi et al., 2002).

Type D is suggested to be an independent psychosocial risk factor for poor cardiac prognosis and increased mortality in CAD patients (Denollet et al., 1996; Denollet, 2000; Denollet et al., 2006a). However, the mechanism underlying these associations is not fully understood, but may include inflammatory processes (Conraads et al., 2006; Denollet et al., 2003; Kupper et al., 2009; van Dooren et al., 2016). To date, no study has examined NADPH oxidase-derived superoxide anion production by monocyte-derived human inflammatory M1 macrophages in CAD patients with Type D. Such a study could provide a further potential hint for a dysregulated immune system, which may explain the association between Type D and poor cardiac prognosis in CAD patients.

In order to shed more light on the mechanisms that link hypertension with an increased risk of atherosclerosis and Type D with poor cardiac prognosis in CAD, respectively, we aimed to investigate whether NADPH oxidase-derived superoxide anion production by human monocyte-derived inflammatory M1 macrophages is higher in essential hypertension and CAD patients with Type D as compared to respective controls. Notably, inflammatory macrophages are pivotal cells in the atherosclerotic process and the main cells presented in atherosclerotic lesions. We hypothesized that both individuals with essential hypertension and

CAD patients with Type D show a higher NADPH oxidase-derived superoxide anion production by inflammatory M1 macrophages compared to controls.

To address these research questions, the use of a simple and appropriate method for the measurement of *phagocytic* NADPH oxidase-derived superoxide anion production by human monocyte-derived inflammatory M1 macrophages was needed. Therefore, we first implemented and validated an *in vitro* method suitable for assessing *phagocytic* NADPH oxidase-derived superoxide anion production by human monocyte-derived inflammatory M1 macrophages, which can be used in psychosomatic or psychobiological research.

4 EMPIRICAL STUDIES

4.1 Summaries of the empirical studies

4.1.1 An in vitro method to investigate microbicidal potential of human macrophages for use in psychosomatic research

Theoretical background: Psychological states relate to changes in circulating immune cells, but as yet, associations with immune cells in peripheral tissues such as macrophages have hardly been investigated. Here, we aimed to implement and validate a method for measuring the microbicidal potential of *ex vivo* isolated human monocyte-derived macrophages as an indicator of macrophage activation.

Methods: The method was implemented and validated for two blood sampling procedures (short-term cannula insertion vs. long-term catheter insertion) in 79 participants (34 women, 45 men) aged between 18 and 75 years. The method principle is based on the reduction of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-dis-ulfophenyl)-2H tetrazolium, monosodium salt (WST-1) by superoxide anions, the first in a series of pathogen-killing reactive oxygen species produced by phorbol-myristate-acetate (PMA)-activated human monocyte-derived macrophages. Cytochrome c reduction and current generation were measured as reference methods for validation purposes. We further evaluated whether depressive symptom severity (Beck Depression Inventory [BDI]) and chronic stress (Chronic Stress Screening Scale [CSSS]) were associated with macrophage microbicidal potential.

Results: The assay induced superoxide anion responses by human monocyte-derived macrophages in all participants. Assay results depended on blood sampling procedure (cannula vs. catheter insertion). Inter-assay variability as a measure for assay reliability was ≤ 10.92%. WST-1 reduction scores correlated strongly with results obtained by reference methods (cytochrome c: $r = .57$, $p = .026$; current generation: $r's \geq .47$, $p's < .033$) and with psychological factors (depressive symptom severity: $r = .35$ [cannula insertion] vs. $r = -.54$ [catheter insertion]; chronic stress: $r = .36$ [cannula insertion]; $p's < .047$).

Discussion: Our findings suggest that the implemented *in vitro* method investigates microbicidal potential of human monocyte-derived macrophages in a manner that is valid and sensitive to psychological measures.

The full manuscript of this study, including detailed information on the theoretical background, the methods, the results, and the discussion, is provided in the Appendix (see A2 on p. 158). This study was published as an original research article in the peer-reviewed journal "*Psychosomatic Medicine*".

Kuebler, U., Ehlert, U., Zuccarella, C., Sakai, M., Stemmer, A. & Wirtz, P.H. (2013). An in vitro method to investigate the microbicidal potential of human macrophages for use in psychosomatic research. *Psychosomatic Medicine*, 75, 841-888.

4.1.2 Macrophage superoxide anion production in essential hypertension: Associations with biological and psychological cardiovascular risk factors

Theoretical background: Essential hypertension is an important risk factor for CAD and its underlying process atherosclerosis, but involved mechanisms are not fully understood. Both macrophages and superoxide anions have been proposed to play a major role in the pathogenesis of atherosclerosis. In the present study, we investigated whether macrophages of individuals with hypertension show higher NADPH oxidase-derived superoxide anion production compared to normotensives. Furthermore, we examined associations between macrophage superoxide anion production and the psychological factors depression and chronic stress independently of hypertension status.

Methods: We studied 30 hypertensive (M: 48.7 ± 2.4 years) and 30 age-matched normotensive men (M: 48.6 ± 2.4 years). We assessed macrophage superoxide anion production using the WST-1 assay. The assay is based on the chemical reduction of the cell-impermeative tetrazolium salt WST-1 by superoxide anions that are produced by activated human *ex vivo* isolated monocyte-derived macrophages. We further evaluated whether chronic stress or depressive symptom severity were associated with macrophage superoxide anion production. All analyses were adjusted for potential confounders.

Results: Individuals with hypertension showed higher superoxide anion production compared to normotensives (F (1,58) = 11.56, p = .001). Complementary analyses using MAP as a continuous measure revealed that higher MAP correlated significantly with higher WST-1

reduction (ß = .38, p= .003, Δ R^2 = .145). These results remained significant when controlling for potential confounding influences. Chronic stress (β = .24, p = .067, ΔR^2= .053), but not depression (p = .24), was independently related to marginally higher WST-1 reduction scores.

Discussion: Our results indicate higher macrophage superoxide anion production in individuals with hypertension compared to normotensives. This may suggest a mechanism underlying cardiovascular risk with hypertension.

The full manuscript of this study, including detailed information on the theoretical background, the methods, the results, and the discussion, is provided in the Appendix (see A3 on p. 187). This study was published as an original research article in the peer-reviewed journal "*Psychosomatic Medicine"*.

Zuccarella-Hackl, C., von Känel, R., Thomas, L., Hauser, M., Kuebler, U., Widmer, H.R. & Wirtz, P.H. (2016). Macrophage superoxide anion production in essential hypertension: associations with biological and psychological cardiovascular risk factors. *Psychosomatic Medicine,* 78, 750-757.

4.1.3 Higher macrophage superoxide anion production in coronary artery disease patients with Type D personality

Theoretical background: Type D personality (Type D) is an independent psychosocial risk factor for poor cardiac prognosis and increased mortality in patients with cardiovascular disease (CVD), but the involved mechanisms are poorly understood. Macrophages play a pivotal role in atherosclerosis, the process underlying coronary artery disease (CAD). We investigated macrophage superoxide anion production in CAD patients with and without Type D.

Methods: We studied 20 male CAD patients with Type D (M: 66.7 ± 9.9 years) and 20 age-matched male CAD patients without Type D (M: 67.7 ± 8.5 years). Type D was measured using the DS14 questionnaire with the two subscales 'negative affectivity' and 'social inhibition'. We assessed macrophage superoxide anion production using the WST-1 assay. All analyses were controlled for potential confounders.

Results: CAD patients with Type D showed higher superoxide anion production compared to CAD patients without Type D ($F(1,38) = 15.57$, $p < .001$). Complementary analyses using the Type D subscales 'negative affectivity' and 'social inhibition', and their interaction as continuous measures, showed that both Type D subscales (negative affectivity: ($ß = .48$, $p = .002$, $R^2 = .227$); social inhibition: ($ß = 46$, $p = .003$, $R^2 = .208$) and their interaction ($ß = .36$, $p = .022$, $R^2 = .130$) were associated with higher WST-1 reduction scores. Results remained significant when controlling for classical CVD risk factors (i.e. body mass index, mean arterial blood pressure), atherosclerosis severity (i.e. intima media thickness, presence of carotid plaques), and psychological factors (depressive symptom severity, chronic stress).

Discussion: Our results indicate higher macrophage superoxide anion production in CAD patients with Type D compared to those without Type D. This may suggest a mechanism contributing to increased morbidity and mortality in CAD patients with Type D.

The full manuscript of this study, including detailed information on the theoretical background, the methods, the results, and the discussion, is provided in the Appendix (see A4 on p. 213). This study was published as an original research article in the peer-reviewed journal "*Psychoneuroendocrinology*".

Zuccarella-Hackl, C., von Känel, R., Thomas, L., Kuebler, P., Schmid, J.P., Mattle, H.P., Mono, M.L., Rieben, R., Wiest, R. & Wirtz, P.H. (2016). Higher macrophage superoxide anion production in coronary artery disease (CAD) patients with Type D personality. *Psychoneuroendocrinology*, 68, 186-193.

5 DISCUSSION

5.1 General Discussion

The research questions of this thesis arose from a variety of animal and human studies indicating that immunological processes, in particular NADPH oxidase and NADPH oxidase-derived superoxide anions, play a crucial role in the pathogenesis and progression of atherosclerosis, and thus CAD. In an attempt to answer the research questions, three experimental studies were conducted, the results of which were summarized in the previous chapter and will be presented and discussed in detail – as original research articles – in the Appendix. In this chapter, the main findings of the studies and the methodological approaches will be discussed and reflected on from a more general perspective. Following this, the thesis concludes with directions for future research.

5.1.1 Discussion of the results and methodological approaches

From numerous studies, it is known that essential hypertension, as a well-established cardiovascular risk factor, increases the risk of atherosclerosis and related CAD (Alexander, 1995; Hajjar et al., 2006; Libby et al., 2002), whereas Type D, as a psychosocial risk factor, increases the risk for poor cardiac prognosis and mortality in CAD patients (Denollet et al., 1996; Denollet, 2000; Denollet et al., 2006). However, underlying mechanisms are still unclear.

Atherosclerosis, as the underlying pathophysiological process of CAD, is defined as a progressive inflammatory process of arterial wall thickening with intense immunological activity (Hansson & Libby, 2006). The inflammatory nature of atherosclerosis raises the possibility to search for inflammatory biomarkers, which may explain the association between hypertension and increased risk of atherosclerosis and Type D and poor cardiac prognosis in CAD, respectively. Indeed, there is evidence of increased inflammatory biomarkers in hypertension and cardiac patients with Type D. In essential hypertension compared to normotensive controls, increased plasma levels of the acute-phase protein CRP and the proinflammatory cytokines TNF-α and IL-6 were found, independent of potential confounding variables such as age, sex, BMI, lipid profile, and positive family history of hypertension (Bautista, Vera, Arenas & Gamarra, 2005; Schillaci et al., 2003). In particular,

higher levels of TNF-α and IL-6 may increase the risk of atherosclerosis under hypertensive conditions (Furumoto, Saito, Dong, Mikami, Fujii & Kitabatake, 2002). Despite this promising evidence, there are also studies reporting no association between TNF-α (Sheu, Lee, Chang & Chen, 2000) or IL-6 (Mirhafez et al., 2014) and hypertension. Therefore, the role of these cytokines in mediating the risk of atherosclerosis in hypertension remains unclear. In cardiac patients with Type D, only a small number of studies have examined the role of the inflammatory biomarkers. These studies reported that Type D in CHF patients independently predicts higher levels of TNF-α and TNF-α soluble receptors, suggesting that Type D is a marker of immune activation. This immune activation, in turn, may possibly mediate the association between Type D and poor cardiac prognosis in CAD. However, a potential mediating role of this cytokine is still not fully understood (Conraads et al., 2006; Denollet et al., 2003).

Inflammatory processes have also been implicated in endothelial dysfunction in hypertension (Fortuño et al., 2004; Zalba et al., 2000; Zalba et al., 2001) and CAD (Guzik et al., 2000a; Heitzer et al., 2001; Judkins et al., 2010). Under normal homeostatic conditions, the endothelium maintains blood circulation and fluidity as well as vascular tone and inflammatory responses (Gonzalez & Selwyn, 2003). However, cardiovascular risk factors such as hypertension lead to an alteration in endothelial function (Gonzalez & Selwyn, 2003), characterized by the inability to maintain vascular homeostasis (Ross, 1999). This condition is termed endothelial dysfunction (Münzel, Groi, Bruno & Taddei, 2010).

Endothelial dysfunction represents an early stage of atherosclerosis (Ross, 1999) and has been documented in individuals with essential hypertension (Hadi, Carr & Suwaidi, 2005). Furthermore, endothelial dysfunction promotes vascular complications (e.g. accelerates atherosclerosis) and has been shown to be an independent predictor of poor cardiac prognosis in CAD patients (Halcox et al., 2002; Heitzer et al., 2001; Suwaidi et al., 2000). Moreover, a very recent study reported an association between Type D and endothelial dysfunction (van Dooren et al., 2016).

The mechanisms by which cardiovascular risk factors produce endothelial dysfunction are complex and multifactorial but may include the production of ROS, in particular superoxide anions, generated by activation of NADPH oxidase complex, as indicated by animal and human studies in hypertension and atherosclerosis / CAD (Münzel et al., 2010). These studies consistently revealed that increased NADPH oxidase-derived superoxide anion production led to a subsequent decrease in vascular NO bioavailability, which probably may result in

endothelial dysfunction. This association was mainly demonstrated in vascular wall cells such as VSMCs (Guzik et al., 2000a; Heitzer et al., 2001; Judkins et al., 2010; Zalba et al., 2000; Zalba et al., 2001), but there is also evidence for this association in phagocytic cells such as neutrophils (Mehta et al., 1994) and macrophage precursor cells (Fortuño et al., 2004).

Besides the implication of NADPH oxidase-derived superoxide anions in endothelial dysfunction, there is a growing body of evidence for an increased NADPH oxidase activity and subsequently increased NADPH oxidase-derived superoxide anion production in hypertension (Cifuentes et al., 2000; Maeda et al., 2003; Modlinger et al., 2006; Rey et al., 2001; Zalba et al., 2000) and atherosclerosis / CAD (Azumi et al., 1999; Guzik et al., 2006; Judkins et al., 2010; Sorescu et al., 2002). This increase in superoxide anion production, mainly generated by NADPH oxidase activation in VSMCs, was associated with an increase in atherosclerotic lesion formation as indicated by animal models of atherosclerosis (Barry-Lane et al., 2001; Vendrov et al., 2007). In contrast, in human atherosclerotic coronary arteries from CAD patients, the increased NADPH oxidase activity and NADPH oxidase-derived superoxide anion production – largely due to higher macrophage infiltration (Guzik et al., 2006; Sorescu et al., 2002) – was highest in the macrophage-rich plaque shoulder (Azumi et al., 1999; Sorescu et al., 2002) and was further associated with higher severity of atherosclerosis (Sorescu et al., 2002). In addition, in individuals with essential hypertension, an increased NADPH oxidase-derived superoxide anion production by circulating macrophage precursor cells was found (Fortuño et al., 2004; Watanabe et al., 2006), which was positively associated with carotid artery IMT, as a vague index for atherosclerosis severity (Watanabe et al., 2006).

Notably, to date, no study has examined the role of NADPH oxidase-derived superoxide anion production in CAD patients with Type D, although a dysregulation of the immune system is proposed to mediate the association between Type D and poor cardiac prognosis in CAD (Conraads et al., 2006; Denollet et al., 2003). However, in CHF patients, Type D has been shown to be associated with increased oxidative stress (Kupper et al., 2009), as characterized by an imbalance between the production of ROS and antioxidant defense mechanisms (Betteridge, 2000). It should be emphasized that in the study by Kupper and colleagues (2009), oxidative stress was measured by assessing levels of oxidative stress markers such as Hsp70 and XO / Hsp70 ratio, but not by assessing ROS levels. Therefore, the role of ROS, and in particular superoxide anions, is still unknown.

Although monocyte-derived inflammatory macrophages – as key cells in the atherosclerotic process (Ley et al., 2011) – are known to represent the main component of atherosclerotic plaques (Gui et al., 2012) and are further characterized by a high ROS-mediated microbicidal activity (Colin et al., 2014; de Oliveira-Junior et al., 2011; Halliwell, 2006; Mosser & Edwards, 2008; Zhang & Wang, 2014), no study has examined NADPH oxidase-derived superoxide anion production by human inflammatory M1 macrophages in either hypertension or CAD patients with Type D. Such studies might provide new insights into the pathophysiological processes that may contribute to the increased risk of atherosclerosis in hypertension and poor cardiac prognosis in CAD patients with Type D, respectively. This lack of research may possibly be due to the absence of a simple method for measuring macrophage superoxide anion production. Therefore, a simple and appropriate *in vitro* method is needed to fill this gap.

In order to achieve this research goal, in the first study, the WST-1 assay was developed (Kuebler, Ehlert, Zuccarella, Sakai, Stemmer & Wirtz, 2013). This assay is based on a method used in a permanent monocytic cell line (i.e. THP-1 cells; Sakai, Vonderheit, Wei, Kuttel & Stemmer, 2009) and was adapted to *ex vivo* isolated human monocyte-derived inflammatory M1 macrophages. The assay principle is based on the chemical reduction of the cell-impermeative tetrazolium salt WST-1 by superoxide anions that are produced by PMA-activated inflammatory M1 macrophages.

To ensure the most effective procedure for inducing NADH oxidase-derived superoxide anion production by inflammatory M1 macrophages, the influence of different cell stimulation agents as well as the influence on different cell numbers were tested in pilot studies. Therefore, different stimuli that initiate cell differentiation (i.e. differentiation from monocytes into inflammatory macrophages by using the combination of LPS, IFN-γ, and TNF-α), cell activation (i.e. activation of the NADPH oxidase in phagocytic cells by using PMA), or both cell differentiation and activation (i.e. using LPS, IFN-γ, TNF-α, and PMA) as well as different cell numbers (i.e. 3.0, 2.5, 2.0, 1.5, and 1.0 x 10^6) were used. Next, reliability and validity of the assay had to be verified. To assess reliability, the inter-assay variability was tested by splitting blood samples either before or after the Ficoll purification procedure. In detail, each blood sample was split into half, resulting in blood samples A and B of the same initial blood sample. Then, the WST-1 assay was started independently in both blood samples A and B. After Ficoll density gradient purification and re-suspension of isolated PBMCs at a density of 2.5 x 10^6, the PBMC suspension of blood sample A was pipetted into

two wells, rendering the duplicate sample A1 and A2 before continuing with the WST-1 assay. Thus, it was possible to determine inter-assay variability by comparing the amount of WST-1 reduction in sample A1 and sample A2 (i.e. reliability after Ficoll purification), as well as in sample B and the mean of sample A1 and A2 (i.e. reliability before Ficoll purification). To test validity, the WST-1 assay was compared firstly with a reference method using cytochrome c as an analogue of WST-1, and secondly with the generation of electrical current, which has been shown to be associated with PMA-induced release of superoxide anions by THP-1-derived macrophages (Sakai et al., 2009). Finally, to investigate the sensitivity of the assay to psychological parameters, the associations of NADPH oxidase-derived superoxide anion production with self-reported chronic stress and depressive symptom severity were examined. These psychological constructs were chosen due to their association with inflammatory biomarkers that may increase the risk of cardiac disease (Dinan, 2009; Gouin, Glaser, Malarkey, Beversdorf & Kiecolt-Glaser, 2012). Notably, because there are indications that the blood sampling procedure (i.e. short-term cannula insertion or long-term venous catheter insertion) may affect NADPH oxidase-derived superoxide anion production (Kuebler et al., 2013), a comparison of these two blood sampling procedures with respect to macrophage superoxide anion production was further needed. Moreover, NADPH oxidase-derived superoxide anion production had to be corrected by post hoc counted macrophage numbers, in order to minimize the possibility that differences in macrophage superoxide anion production may be related to differences in the number of macrophages derived from the same number of macrophage precursor cells.

Results from this study indicate that the WST-1 assay is a reliable and valid *in vitro* method for assessing NADPH oxidase-derived superoxide anion production by inflammatory M1 macrophages. The associations between NADPH oxidase-derived superoxide anion production and both psychological parameters found in this study further indicate that this *in vitro* assay is of interest to psychosomatic or psychobiological research, and may provide new insights into the pathophysiological process linking psychological risk factors with adverse health outcomes.

Despite these promising findings, some points of critique should be taken into account: First, the NADPH oxidase-derived superoxide anion production measured by means of the WST-1 assay only gives first indications for possible associations with macrophage microbicidal activity. Second, the implementation of the WST-1 assay was only carried out by using WST-1 reduction as a detection method for NADPH oxidase-derived superoxide anion production

by inflammatory M1 macrophages, and not by using other detection methods such as chemiluminescence or flow cytometry methods. Third, because no specific exclusion criteria were applied in participants using the short-term cannula insertion as blood sampling procedure, sex differences or other potential unknown confounders may bias results.

The successful implementation and validation of the WST-1 assay (study 1) provided the foundation upon which to examine the central aim of this thesis; namely, to shed more light on pathophysiological processes that may contribute to increased risk of atherosclerosis in hypertension (study 2) and increased risk of poor cardiovascular prognosis in CAD patients with Type D (study 3), respectively. Studies 2 and 3 were part of a larger project examining psychoneurobiological mechanisms in essential hypertension and CAD. For the purpose of the second study, we recruited 30 apparently healthy, nonsmoking, and medication-free men with essential hypertension and 30 normotensive men, whereas for the purpose of the third study, 20 male patients with CAD and Type D and 20 male patients with CAD but without Type D were recruited. In order to balance the study groups with regard to age, we chose an age-matching procedure on a case-by-case basis. In detail, for each of the individuals with essential hypertension or CAD patients with Type D, we recruited an age-matched normotensive control or an age-matched CAD patient without Type D, respectively. This procedure allowed us to rule out possible confounding effects of age on the results, because the groups were considered independent, with the main effect of age being balanced. To further reduce potential confounding factors, all analyses were controlled for a priori selected traditional risk factors (e.g. BMI) and for psychological risk factors (i.e. depressive symptom severity, chronic stress), which have been shown to be associated with immune activation or NADPH oxidase-derived superoxide anion production by inflammatory M1 macrophages. The analyses in CAD patients were further adjusted for atherosclerotic disease severity parameters (i.e. IMT, presence of plaques).

Results indicate that NADPH oxidase-derived superoxide anion production by inflammatory M1 macrophages was higher in individuals with essential hypertension and CAD patients with Type D compared to their respective control groups. These results were independent of the included potential confounding factors. In addition, in study 2, higher MAP was independently associated with higher NADPH oxidase-derived superoxide anion production, whereas in study 3, both Type D subscales NA and SI as well as their interaction were independently associated with higher macrophage superoxide anion production. These

findings imply that inflammatory M1 macrophages of individuals with essential hypertension and CAD patients with Type D seem to show an increased preparedness to kill microbes in reaction to stimulating agents. Importantly, these associations were linear in nature, i.e. the preparedness rose with increasing MAP and increasing NA, SI, and their interaction, respectively. Thus, these results suggest that NADPH oxidase-derived superoxide anion production by inflammatory M1 macrophages may play a mechanistic role in the mediation of both atherosclerotic risk in hypertension and poor cardiac prognosis in CAD patients with Type D.

However, despite these promising results, it should be mentioned that these studies were cross-sectional, and assumptions regarding causality remain unclear, although it seems reasonable to assume that both essential hypertension and Type D in CAD patients increase NADPH oxidase-derived superoxide anion production by inflammatory M1 macrophages, which in turn may lead to increased atherosclerotic risk and poor cardiac prognosis, respectively. Furthermore, the studies focused on male individuals with essential hypertension or male CAD patients with and without Type D, and may therefore not be generalized to women or patients with other diseases. Moreover, the sample size in both studies was small and the number of potential confounding variables was restricted in order to avoid overcontrolling. Therefore, it cannot be ruled out that unmeasured potential confounders may have an impact on NADPH oxidase-derived superoxide anion production by inflammatory M1 macrophages and thus on the observed findings.

In sum, the implementation and validation of the WST-1 assay, as a new, simple, and accurate method for assessing NADPH oxidase-derived superoxide anion production by inflammatory M1 macrophages, offered the possibility to provide new insights into potential underlying pathophysiological mechanisms relating to increased risk of atherosclerosis in hypertension and poor cardiac prognosis in CAD patients with Type D. However, the exact mechanisms underlying the observed higher NADPH oxidase-derived superoxide anion production in hypertension and CAD patients with Type D remain unclear. Based on several prospective human studies reporting that inflammatory processes may be predictive for the development of hypertension (Engström, Lind, hedblad, Stavenow, Zanzon & Lindgärde, 2002; Niskanen et al., 2004; Sesso, Buring, Rifai, Blake, Gaziano & Ridker, 2003) and may further be a potential biological basis of poor cardiac prognosis in CAD patients with Type D (Conraads et al., 2006; Denollet et al., 2003; Kupper et al., 2009; van Dooren et al., 2016), it might be

possible that inflammatory processes prime inflammatory M1 macrophages to produce a greater amounts of NADPH oxidase-derived superoxide anions. This increased release of NADPH oxidase-derived superoxide anions may further promote vascular wall injuries, which may eventually result in atherosclerotic events.

5.1.2 Directions for future research

Data from this thesis indicate that the WST-1 assay is a reliable and valid *in vitro* method for assessing NADPH oxidase-derived superoxide anion production by human inflammatory M1 macrophages. Furthermore, with the use of this assay, it was possible to obtain new insights into potential pathophysiological processes that underlie increased risk of atherosclerosis in hypertension and poor cardiac prognosis in CAD patients with Type D. However, more evidence is needed.

Given the small sample sizes used in our studies and the restriction of our results to apparently healthy, nonsmoking, and medication-free hypertensive and normotensive men, as well as male CAD patients with Type D, in a first step, future studies should replicate these findings with larger sample sizes as well as different subpopulations (e.g. women, patients with other forms of cardiovascular disease) in order to enhance generalizability. These studies should additionally explore associations between further biological (e.g. blood lipids, age, BMI) and psychological (e.g. lack of social support, major depression) cardiovascular risk factors as well as parameters of atherosclerosis severity (e.g. IMT, presence of carotid plaques) using a cross-sectional and longitudinal study design. In a second step, intervention studies might be conducted. These studies should investigate whether a medication-induced lowering of BP or a reduction of Type D scores, respectively, reduce NADPH oxidase-derived superoxide anion production by human inflammatory M1 macrophages.

To allow causal conclusions from our results, prospective investigations are needed which allow the examination of associations between the presence and absence of potential risk factors and the development of a disease. Referring to our results, a prospective study could basically be conducted as follows: Macrophage superoxide anion production of healthy individuals and CAD individuals without Type D personality are measured at baseline. Based on these measurements, participants are classified into four groups (i.e. healthy individuals / CAD patients without Type D with high macrophage superoxide anion production and

healthy individuals / CAD patients without Type D with low macrophage superoxide anion production). Following this, all participants are followed longitudinally over a period of years, that is, all parameters of interest are measured after several a priori defined follow-up intervals. Thus, macrophage superoxide anion production can be studied as a potential risk factor for the development of hypertension in healthy participants and Type D in CAD patients, respectively.

Notably, a further important extension in future research could also refer to the additional assessment of proinflammatory cytokines released by inflammatory M1 macrophages. This could be beneficial because inflammatory processes have been reported to be predictive for the development of hypertension (Engström et al., 2002; Niskanen et al., 2004; Sesso et al., 2003) and have been discussed as a potential biological basis of poor cardiac prognosis in CAD patients with Type D (Conraads et al., 2006; Denollet et al., 2003; Kupper et al., 2009; van Dooren et al., 2016). In addition, the proinflammatory cytokine TNF-α generated by activated macrophages is suggested to serve as a regulator of the NADPH oxidase, resulting in an increased and / or prolonged production of superoxide anions (Gauss et al., 2007). An extension in this regard would help to gain a better understanding of the underlying mechanisms that link hypertension with increased risk of atherosclerosis as well as poor cardiac prognosis with CAD and Type D, and is further essential for the development of effective treatments.

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7 APPENDIX

A1: DS14

Nachstehend finden Sie einige Aussagen, die Menschen häufig verwenden, um sich selbst zu beschreiben. Bitte lesen Sie jede Aussage und kreuzen Sie daneben die für Sie passende Antwort an! Es gibt keine richtigen oder falschen Antworten: Nur Ihr eigener Eindruck zählt!

Bitte geben Sie zunächst an, wie Sie sich *üblicherweise* oder *im Allgemeinen* einschätzen! **Im Allgemeinen gilt für mich:**	trifft überhaupt nicht zu	trifft eher nicht zu	unentschieden	trifft eher zu	trifft voll und ganz zu
1. Es fällt mir leicht, Kontakt mit anderen Menschen zu knüpfen					
2. Ich rege mich oft über unwichtige Dinge auf					
3. Ich unterhalte mich oft mit Fremden					
4. Ich fühle mich oft unglücklich					
5. Ich bin oft gereizt					
6. Ich fühle mich oft im Umgang mit Anderen gehemmt					
7. Ich sehe die Dinge pessimistisch					
8. Es fällt mir schwer, mit Anderen ein Gespräch zu beginnen					
9. Ich bin oft schlechter Laune					
10. Ich bin vom Wesen her verschlossen					
11. Ich neige dazu, andere Leute auf Abstand zu halten					
12. Ich mache mir oft Sorgen					
13. Ich bin oft schlecht drauf					
14. Ich weiss nicht, worüber ich mit Anderen reden soll					

Möglicherweise geht es Ihnen zur Zeit besser oder schlechter als üblich. **In der Folge beurteilen Sie daher bitte, inwieweit die folgenden Aussagen *in der letzten Woche* (einschließlich heute) auf Sie zutrafen:**	traf überhaupt nicht zu	traf eher nicht zu	unentschieden	traf eher zu	traf voll und ganz zu
15. Es fiel mir leicht, Kontakt mit anderen Menschen zu knüpfen					
16. Ich habe mich oft über unwichtige Dinge aufgeregt					
17. Ich habe mich oft mit Fremden unterhalten					
18. Ich war unglücklich					
19. Ich war oft gereizt					
20. Ich habe mich im Umgang mit Anderen gehemmt gefühlt					
21. Ich war pessimistisch					
22. Es fiel mir schwer, mit Anderen ein Gespräch zu beginnen					
23. Ich war oft schlechter Laune					
24. Ich fühlte mich verschlossen					
25. Ich wollte andere Leute lieber auf Abstand halten					
26. Ich habe mir viele Sorgen gemacht					
27. Ich war schlecht drauf					
28. Ich wusste nicht, worüber ich mit Anderen reden sollte					

A2: AN IN VITRO METHOD TO INVESTIGATE MICROBICIDAL POTENTIAL OF HUMEN MACROPHAGES FOR USE IN PSYCHOSOMATIC RESEARCH

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Abstract

Objective: Psychological states relate to changes in circulating immune cells, but associations with immune cells in peripheral tissues such as macrophages have hardly been investigated yet. Here, we aimed to implement and validate a method for measuring the microbicidal potential of *ex vivo* isolated human monocyte-derived macrophages (HMDM) as an indicator of macrophage activation.

Methods: The method was implemented and validated for two blood sampling procedures (short-term cannula insertion vs. long-term catheter insertion) in 79 participants (34 women, 45 men) aged between 18 and 75 yrs. The method principle is based on the reduction of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-dis-ulfophenyl)-2H tetrazolium, monosodium salt (WST-1) by superoxide anions, the first in a series of pathogen-killing reactive oxygen species produced by phorbol-myristate-acetate (PMA)-activated HMDM. Cytochrome c reduction and current generation were measured as reference methods for validation purposes. We further evaluated whether depressive symptom severity (Beck Depression Inventory [BDI]) and chronic stress (Chronic-Stress-Screening-Scale [CSSS]) were associated with macrophage microbicidal potential.

Results: The assay induced superoxide anion responses by HMDM in all participants. Assay results depended on blood sampling procedure (cannula vs. catheter insertion). Inter-assay variability as a measure for assay reliability was ≤ 10.92%. WST-1 reduction scores correlated strongly with results obtained by reference methods (cytochrome c: r = .57, p = .026; current generation: r's ≥ .47, p's < .033) and with psychological factors (depressive symptom severity: r = .35 [cannula insertion] vs. r = -.54 [catheter insertion]; chronic stress: r = .36 [cannula insertion]; p's < .047).

Conclusions: Our findings suggest that the implemented *in vitro* method investigates microbicidal potential of HMDM in a manner that is valid and sensitive to psychological measures.

1 INTRODUCION

A growing body of psychosomatic research documents that psychological states are linked to quantitative and qualitative alterations in circulating immune cells (e.g. Ader, 2006; Rabin, 1999). In contrast, relatively little is known about associations between psychological states

and alterations in immune cells in peripheral tissues such as macrophages (mature, tissue-differentiated monocytes). Activated macrophages are important effector cells involved in warding off microorganisms and regulating inflammation (Taylor et al., 2005). We recently found first evidence that acute stress relates to alterations in wound-induced macrophage activation but associations with longer-lasting psychological states without prior wound-induction have not yet been investigated (Kuebler et al., 2013). The lack of research in this field may be related to both the absence of and familiarity with methods to analyze macrophage activity. Given that macrophages are primarily activated to develop microbicidal effector functions (Schroder et al., 2004), the purpose of our study was to systematically validate an *in vitro* method suitable for assessing microbicidal potential of human macrophages, which may be used in future psychosomatic and psychobiological research.

Microbicidal effectiveness of human macrophages is due in large part to the production of reactive oxygen species (ROS; de Oliveira-Junior et al., 2011). ROS production is mediated by activation of the membrane-bound enzyme complex nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Once activated, NADPH oxidase transfers electrons from NADPH in the cytosol to extracellular or intraphagolysosomal oxygen molecules. These oxygen molecules are then chemically reduced to superoxide anions, a type of ROS (de Oliveira-Junior et al., 2011). The superoxide anions formed in this reaction serve as precursors to other, more reactive ROS such as hydrogen peroxide and hypochlorous acid (El-Benna et al., 2005). The assay presented here to study the microbicidal potential of human macrophages is an adaptation of a method used in a permanent monocytic cell line (THP-1 cells) as described by Sakai and colleagues (2009). To allow exploration of associations with psychological states, we adapted the existing method to *ex vivo* isolated human monocyte-derived macrophages.

The assay principle is based on the chemical reduction of the cell-impermeative tetrazolium salt WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt) by superoxide anions that are produced by phorbol myristate acetate (PMA)-activated human monocyte-derived macrophages (HMDM). HMDM represent the classically activated (M1) macrophage also known as the inflammatory macrophage phenotype (Martinez et al., 2008). More specifically, human blood monocytes are isolated from whole blood and then differentiated *in vitro* into inflammatory macrophages. Inflammatory macrophages are characterized by high cytosolic expression of NADPH oxidase, the prerequisite for high oxygen-dependent microbicidal activity (Dale et al., 2008).

To induce macrophage microbicidal activity intended to destroy pathogens, NADPH oxidase needs to be activated. Activation of NADPH oxidase can be experimentally induced by *in vitro* incubation with PMA (for details see Cathcart, 2004). Once activated, the NADPH oxidase catalyzes the reduction of oxygen to superoxide anions, which in turn reduce WST-1 (Tan & Berridge, 2000; Sakai et al., 2009). The reduction of WST-1 results in the formation of a colored, water-soluble formazan salt with increased absorbance at 450 nm (Sakai et al., 2009; Tan & Berridge, 2000). Consequently, the colorimetric measurement of formazan formation in the medium in which macrophages are suspended indicates superoxide anion-induced WST-1 reduction and thus macrophage (NADPH oxidase-mediated) microbicidal potential.

In the present study, the assay implementation and validation process was as follows: First, we tested the influence of various stimulation agents on superoxide anion production from human inflammatory macrophages as well as the influence of different cell numbers on WST-1 reduction scores in pilot studies. Second, to ensure assay reliability, we investigated the inter-assay variability in a sample of men and women. Additionally, to ensure validity, we conducted the assay and compared results with reduced cytochrome c as a reference method. Next, in order to investigate sensitivity of the WST-1 macrophage assay to psychological states, we tested for associations of macrophage microbicidal potential with self-reported chronic stress and depressive symptoms severity. Notably, all blood samples for performing the WST-1 macrophage assay were collected by short-term cannula insertion (cannula group). Since we recently found first indications that the blood sampling procedure (i.e. short-term cannula insertion or long-term venous catheter insertion) seems to affect macrophage microbicidal potential (Kuebler et al., 2013), we additionally aimed to compare WST-1 reduction scores based on blood samples obtained by short-term cannula insertion with WST-1 reduction scores based on blood samples obtained by venous catheter inserted long enough to start wound healing processes (Mahdavian Delavary et al., 2011). For this purpose, in a third sample of healthy men blood samples were collected via an indwelling venous catheter inserted 2 hours before the WST-1 macrophage assay was performed (catheter group). We additionally validated the WST-1 macrophage assay in the catheter group by comparing WST-1 reduction scores with current generation as a reference method, and tested for associations between the WST-1 macrophage assay and depressive symptom severity.

2 MATERIALS AND METHODS

2.1 Reagents and chemicals

We used the following reagents: Ficoll-Paque PLUS (Ficoll; no. 17-1440-02; GE Healthcare; Uppsala, Sweden); 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1; no. 150849-52-8, Dojindo Laboratories; Kumamoto, Japan); Interferon-γ (IFN-γ; no. PHC4031, Invitrogen; Basel, Switzerland), tumor necrosis factor-α (TNF-α; no. PHC3016, Invitrogen; Basel, Switzerland); Hank's balanced salt solution without phenol red (HBSS; no. 14025050, Invitrogen; Basel, Switzerland); fetal bovine serum (FBS; no. 10106-169, Invitrogen; Basel, Switzerland); Lipopolysaccharide (LPS; no.L6529, Sigma-Aldrich; Buchs, Switzerland); phosphate buffered saline (PBS; no. P5368, Sigma-Aldrich; Buchs, Switzerland); phorbol 12-myristate 13-acetate (PMA; no. P8139, Sigma-Aldrich; Buchs, Switzerland); cytochrome c (no. C2037, Sigma-Aldrich; Buchs, Switzerland); RPMI-1640 Media with Glutamax (RPMI-1640; no. W9925E, Fisher Scientifc; Wohlen, Switzerland); Diff-Quick (no. 130832; Siemens, Zurich, Switzerland).

2.2 Participants

The assay validation study was performed in three different samples and was conducted from January 2010 until March 2013.

2.2.1 Study sample 1

Study sample 1a consisted of 11 apparently healthy Caucasian adults (6 women, 5 men) aged between 23 and 34 years (mean age ± SEM; 27 ± 1.2 years) who provided one blood sample each. Study sample 1b consisted of four apparently healthy Caucasian women. Analogous to the subsequent experiments described below, we distinguished between blood sampling procedures: While blood samples were taken by short-term cannula insertion (cannula group) in study sample 1a, in study sample 1b blood samples were taken by an indwelling venous catheter inserted two hours before blood sampling (catheter group). The blood samples of study sample 1b were provided on six different days with assays run in triplicates on three days rendering a total of 12 measurements.

2.2.2 Study sample 2 (cannula group)

The study sample consisted of 40 Caucasian adults (24 women, 16 men) aged between 18 and 75 years (mean age ± SEM; 40 ± 3.1 years). Table 1 provides characteristics of this study

sample. While female subjects were recruited via advertisements from the Canton of Zurich as part of an ongoing study on the effects of psychological stress on health outcomes, male subjects were recruited via advertisements from the Canton of Bern as part of an ongoing study assessing psychobiological mechanisms in hypertension and coronary heart disease. No specific exclusion criteria were applied.

Each participant provided a blood sample taken by short-term cannula insertion and completed the Beck Depression Inventory (BDI) as well as the Chronic Stress Screening Scale (CSSS; see below). The study protocol was formally approved by the Ethics Committee of the University of Zurich, Switzerland, and by the Ethics Committee of the Canton of Bern, Switzerland. All subjects provided written informed consent.

2.2.3 Study sample 3 (catheter group)

We recruited 24 healthy, medication-free, non-smoking Caucasian men aged between 20 and 48 years (mean age ± SEM; 37 ± 1.5 years). Subjects' characteristics are depicted in Table 1. Study participants were in good physical and mental health as confirmed by telephone interview. Explicit exclusion criteria were: regular strenuous exercise, smoking, alcohol and illicit drug abuse, any heart disease, varicosis or thrombotic diseases, elevated blood sugar and diabetes, elevated cholesterol, liver and renal diseases, chronic obstructive pulmonary disease, allergies and atopic diathesis, rheumatic diseases, and current infectious diseases. If the personal or medication history was not conclusive, the participants' primary care physician was contacted for verification.

Participants were recruited via advertisements and with the help of the Swiss Red Cross of the Canton of Zurich. Each participant provided a blood sample taken at approximately 1:30 p.m. by an indwelling venous catheter inserted two hours before blood sampling and completed the BDI (see below). Notably, we previously used this catheter-insertion procedure as an open-wound paradigm to preactivate monocytes (Kuebler et al., 2013). The study protocol was formally approved by the Ethics Committee of the Canton of Zurich, Switzerland, and written informed consent was obtained from all participants.

2.3 The WST-1 macrophage assay, an *in vitro* method for assessing macrophage NADPH oxidase-mediated microbicidal potential

The method used in this study to measure macrophage superoxide anion production from *ex vivo* isolated human cells is an adaptation of a method used in a permanent monocytic cell line (THP-1 cells) as described by Sakai and colleagues (2009).

2.3.1 Monocyte isolation by adherence after Ficoll purification of peripheral blood mononuclear cells (PBMCs)

In order to separate the cells of interest, nine milliliters of blood were collected in EDTA-coated tubes (Sarstedt, Numbrecht, Germany), immediately layered on top of 10 ml Ficoll (density-based cell separation medium) and centrifuged for 20 minutes at 300 g and 20°C. After centrifugation, peripheral blood mononuclear cells (PBMCs, i.e. lymphocytes and monocytes), were removed from the interface, washed twice in RPMI-1640 medium, counted with a hematologic analyzer (KX-21N; Sysmex Digitana AG), and re-suspended to a concentration of 2.5 x 10^6/ml with RPMI-1640 media supplemented with 10% FBS. Then, PBMC suspension aliquots of 1 ml were transferred to 24-well cell culture plates (no. 4609; Semadeni; Ostermundigen, Switzerland). After incubation for 1 h at 37°C and 5% CO_2, the supernatant was discarded and the plate surface was rinsed five times with 1 ml of warm (25°C) 0.01M PBS to remove non-adherent PBMCs, while monocytes remained adherent to the bottom of the plates. This monocyte isolation method is a well-established procedure to yield monocyte cultures of more than 90% purity (Geng & Hansson, 1992; Mach et al., 1997; Pawlowski et al., 1985; Pawlowski et al., 1983; Selvan et al., 1997).

2.3.2 Differentiation of human monocytes into macrophages and their separation

The resulting adherent monocyte layer (obtained as described above) was diluted with 1 ml RPMI-1640 media supplemented with 10% FBS. Subsequently, we added 2 µl IFN-γ, 2 µl TNF-α, and 0.5 µl LPS resulting in a final concentration of 20 ng/ml IFN-γ, 20 ng/ml TNF-α, and 300 ng/ml LPS to promote differentiation of monocytes into inflammatory macrophages (Ma et al., 2010; Pelegrin & Surprenant, 2009; Taylor et al., 2005). After incubation for 48 h at 37°C and 5% CO_2, the supernatant was discarded and the adherent macrophage layer was washed three times with 1 ml of warm (25°C) 0.01M PBS to remove traces of culture media and non-adherent cells.

2.3.3 WST-1 assay to determine macrophage superoxide anion production

Next, the resulting macrophage monolayer (obtained as described above) was overlaid with 1 ml HBSS buffer solution. Subsequently, 2 µl WST-1, 0.5 µl LPS, 2 µl IFN-γ, 2 µl TNF-α, and 0,5 µl PMA were added, resulting in a final concentration of 100 µM WST-1, 300 ng/ml LPS, 20 ng/ml IFN-γ, 20 ng/ml TNF-α, and 50 nM PMA. This was followed by an incubation period of 4 hours at 37°C and 5% CO_2. Then, the supernatant was removed and used to determine WST-1 reduction by reading the absorbance with a spectrophotometer (SmartSpec Plus, Bio-Rad Laboratories, Inc., Hemel Hempstead, United Kingdom for study samples 1 and 3; Synergy HT, BioTek, Luzern, Switzerland, for study sample 2) at 450 nm against water as blank. Higher optical densities (ODs) as obtained by absorbance reading are associated with higher amounts of WST-1 reduction and thus of superoxide anions generated by HMDM.

2.4 Assay implementation and validation procedures

2.4.1 Identification and verification of macrophage superoxide anion production stimulating agents (study sample 1)

In order to verify the applicability of the cell-line tested *in vitro* method for *ex vivo* isolated HMDM, we tested the influence of different stimuli on the superoxide anion production of *ex vivo* isolated human monocytes / macrophages. Following Sakai and colleagues (2009), we used stimuli, which initiate either cell differentiation; cell activation or both cell differentiation and activation. For cell differentiation, i.e. differentiation of monocytes into inflammatory macrophages, we used the combination of LPS, IFN-γ, and TNF-α; for cell activation, i.e. activation of the NADPH oxidase, we used PMA. Combined differentiation and activation included the use of LPS, IFN-γ, TNF-α, and PMA (detailed protocol in Section 2.3). The stimulation experiments were performed in the 12 subjects of study sample 1a (cannula group) and 12 blood samples of study sample 1b. One female subject of study sample 1a, however, had to be excluded due to problems with venipuncture rendering a final study sample of 11 (6 women, 5 men) for stimuli testing.

2.4.2 Cell-number dependent WST-1 reduction (study sample 2, "cannula group")

In order to obtain pilot data regarding the minimum cell concentration of PBMCs needed to induce measurable PMA-induced superoxide anion release by HMDM we performed the WST-1 macrophage assay using five different PBMC concentrations: 3.0, 2.5, 2.0, 1.5, and

1.0 x 10^6 PBMCs. Experiments were carried out in cells obtained from five female subjects of study sample 2.

2.4.3 Determination of assay reliability (study sample 2, "cannula group")

Assay reliability was assessed by testing inter-assay variability of blood samples split either before or after Ficoll purification. For details on reliability testing see Supplemental Digital Content (SDC) 1. Assay reliability was tested in 24 subjects of study sample 2. One female subject, however, had to be excluded due to technical problems rendering a final study sample of 23 (18 women, 5 men) for reliability testing.

2.4.4 Assay validation by comparison with reference method

2.4.4.1 Validation of the WST-1 macrophage assay by cytochrome c (study sample 2, "cannula group")

Like WST-1, cytochrome c is reduced by superoxide anions (Babior, Kipnes & Curnutte, 1973) and can therefore be used analogous to WST-1 as a detector for superoxide anion production. We thus measured the amount of reduced cytochrome c to validate the WST-1 macrophage assay. For details on this reference method see SDC 2. Assay validation using cytochrome c as reference method was performed in the first 15 participants of study sample 2 (11 women, 4 men).

2.4.4.2 Validation of macrophage superoxide anion production by generation of electrical current (study sample 3, "catheter group")

Current generation observed in the biofuel cell developed by Sakai and colleagues (2009) primarily originates from PMA-induced *superoxide anion* release by THP-1-derived macrophages. The greater *superoxide anion* release, the greater current generation. Therefore, the quantity of electrical power produced in this biofuel cell setup represents a suitable criterion for the validation of the WST-1 macrophage assay. For details on this reference method see SDC 3. Assay validation with this reference method was carried out in 21 subjects of study sample 3.

2.4.5 Macrophage number corrected WST-1 reduction (study sample 2, "cannula group")

Our WST-1 assay principle is based on monocytes included in 2.5 x 10^6 PBMCs that we stimulate by incubation with different agents to differentiate into macrophages. Thus, possible

differences in WST-reduction scores may relate to differences in the number of macrophages derived from that same number of PBMCs. Notably, the same number of PBMCs may result in different numbers of adherent monocyte-derived macrophages. Consequently, we aimed to determine the number of adherent cells per well as an indicator of the final macrophage number per well. We corrected WST-1 reduction score per 2.5 x 10^6 PBMCs (i.e. total WST-1 reduction scores) by post-hoc counted macrophage numbers to obtain a second measure of WST-1 reduction, i.e. macrophage number corrected WST-1 reduction score (corrected WST-1 reduction score). For details on the cell-counting method see SDC 4. The number of adherent cells was determined in the first 30 participants of study sample 2 (15 women, 15 men).

2.5 Psychological assessment

To test for associations between microbicidal potential and psychological factors, the validated German versions of the following self-report questionnaires were used.

2.5.1 Depressive symptom severity (study sample 2 and study sample 3)

Depressive symptom severity was assessed with the 21-item Beck Depression Inventory (BDI), where scores ≥ 10 indicate possible clinical depression (Hautzinger et al., 1994). The BDI was developed for the assessment of depressive symptoms that correspond to the Diagnostic and Statistical Manual of Mental Disorders-IV criteria for major depressive disorders and measures a somatic and a cognitive-affective dimension of depression (Hautzinger et al., 1994). The BDI assesses the frequency and/or severity of symptoms related to sadness, feelings of guilt, perceptions of self-worth, suicidal ideation, and changes in appetite and body weight, among other characteristics. Items have a 4-point scale ranging from 0 (symptom not present) to 3 (symptom very present). Higher scores mean higher depressive symptom severity.

2.5.2 Chronic stress (study sample 2, "cannula group")

Chronic stress was measured using the 12-item Chronic Stress Screening Scale (CSSS) of a larger chronic stress questionnaire (Schulz et al., 2004). The CSSS assesses the frequency of experiencing work overload (4 items), worries (4 items), lack of social recognition (2 item), excessive demands at work (1 item), and social overload (1 item). Items have a 5-point rating

format reflecting frequency (ranging from 1 [never] to 5 [very often]). Higher scores mean higher chronic stress.

2.7 Statistical analysis

Data were analyzed using SPSS Inc. version 19.0 for Windows (Statistical Package for the Social Sciences, SPSS, Chicago, IL, USA) and presented as mean ± *SEM*. All tests were 2-tailed with the level of significance set at $p < .05$. Body mass index (BMI) was calculated by the formula weight in kg/(height in m)^2. WST-1 scores were corrected for macrophage numbers: "corrected WST-1 reduction" refers to WST-1 reduction scores per 10 000 macrophages.

Difference testing. We used univariate analyses of variance (ANOVAs) to test for differences (1) between cannula and catheter group (Table 1, group characteristics), (2) between men and women of the cannula group, (3) between the four stimulation conditions (testing of stimulating agents, study sample 1), and (4) between the five cell number conditions (cell-number dependent WST-1 reduction, study sample 2).

Reliability testing. We determined assay reliability by calculating the mean percentage difference between samples A1 and B (i.e. before Ficoll purification) and between samples A1 and A2 (i.e. after Ficoll purification).

Validity testing. Pearson's correlations were used to validate superoxide anion induced macrophage WST-1 reduction against the reference methods, i.e. cytochrome c reduction and superoxide anion induced macrophage current generation. Since current generation was monitored continuously, we extracted two indices to reflect power generation from the four-hour recording period: first the maximum current value registered (C_{Max}) and then the sum of all current values (C_{Sum}).

Association testing. To test whether psychological factors are associated with the WST-1 macrophage assay correlations were calculated between psychological factors (i.e. BDI and CSSS) and total or corrected WST-1 reduction scores adjusting for age, BMI, and gender (where applicable) as a priori selected control variables because of known alterations in a variety of immune functions with age, BMI and gender (Dorshkind et al., 2009; Schuurs & Verheul, 1990; Wirtz et al., 2008). Unadjusted correlations are presented as well.

3 RESULTS

3.1 Group characteristics (study sample 2, study sample 3)

Table 1 provides the characteristics of study sample 2 (cannula group; n = 40) and study sample 3 (catheter group; n = 24). The two groups did not significantly differ in terms of age and BMI. The catheter group had lower BDI scores than the cannula group and subgroup analysis restricted to men did not change this result (6.0 ± 1.3 [cannula group] vs. 3.2 ± 0.7 [catheter group]; p = .044). In addition, total WST-1 reduction scores were higher in the catheter group than in the cannula group. Within in the cannula group, men and women did not significantly differ in total or corrected WST-1 reduction scores (p's > .62) independent of age and BMI.

Table 1. Group characteristics of participants of study sample 2 and study sample 3

	Study sample 2 (cannula group) n = 40	Study sample 3 (catheter group) n = 24	P-value
Gender (n; men/ women)	16 / 24	24 / 0	
Age (yr)	40.2 ± 3.1	37.1 ± 1.5	.46
BMI (kg/m^2)	24.1 ± 0.6	25.8 ± 1.0	.15
Total WST-1 (OD)	0.070 ± 0.007	0.283 ± 0.021	<.001
Corrected WST-1 (OD)	0.040 ± 0.008	-	
Chronic stress (CSSS)	22.9 ± 2.0	-	
Depressive symptom severity (BDI)	6.9 ± 1.1	3.2 ± 0.7	.022

Notes: Values are means ± SEM. BMI = body mass index; OD = optical density; CSSS = chronic stress screening scale; BDI = beck depression inventory; n = number of participants.

3.2 WST-macrophage assay implementation and validation

3.2.1 Pilot data

3.2.1.1 Identification and verification of macrophage superoxide anion production stimulating agents (study samples 1a and 1b)

WST-1 reduction scores differed significantly between the four stimulation procedures (cannula group: F (3, 43) = 12.78, $p < .001$, $n = 12$; catheter group: F (3, 47) = 347.72, $p < .001$, $n = 11$; Figure 1A). Post-hoc calculated univariate ANOVAs revealed that monocytes / macrophages exposed to both differentiation and activation agents showed the greatest WST-1 reduction capacity compared to cells treated with either none of the stimulation agents (cannula group: F (1, 21) = 23.7, $p < .001$; catheter group: F (1, 23) = 492.54, $p < .001$; negative control) or differentiation (cannula group: F (1, 21) = 16.91, $p = .001$; catheter group: F (1, 23) = 443.28, $p < .001$) and activation (cannula group: F (1, 21) = 4.95, $p = .038$; catheter group: F (1, 23) = 331.45, $p < .001$) agents alone.

The amount of WST-1 reduction reflects NADPH oxidase-derived superoxide anion production by HMDM. These results indicate that stimulation by combined cell differentiating and activating agents constitutes the most effective procedure for inducing superoxide responses.

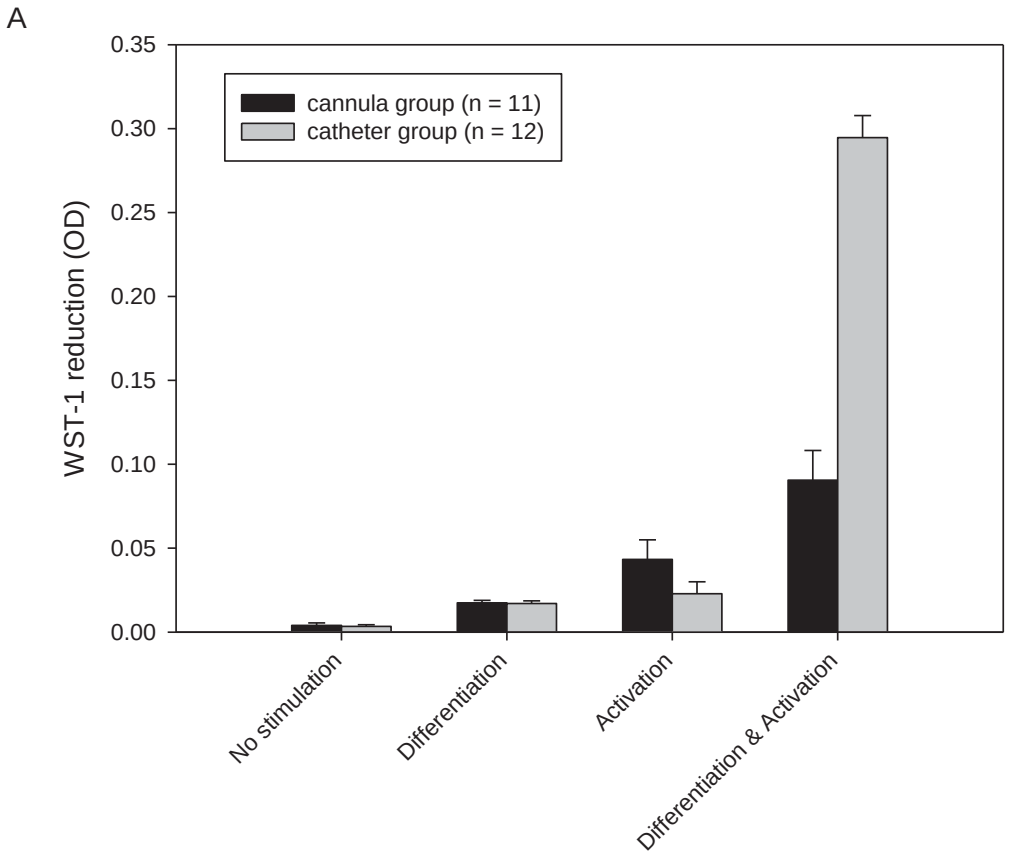

Figure 1A. WST-1 reduction scores between the four stimulation procedures.

Pilot data. Values are given as mean ± *SEM*. Figure 1A depicts WST-1 reduction by *ex vivo* isolated human monocyte-derived macrophages after differential stimulation in a cannula and a catheter group. From the left: Non-stimulated cells as negative control; activation with 50 nM PMA; differentiation with 300 ng/ml LPS, 20 ng/ml IFN-γ, and 20 ng/ml TNF-α; differentiation and activation stimuli (300 ng/ml LPS, 20 ng/ml IFN-γ, 20 ng/ml TNF-α, 50 nM PMA). Across all measurements the combined differentiation and activation stimulation condition differed significantly from all of the other conditions (*p's* ≤ .038).

3.2.1.2 Cell-number dependent WST-1 reduction (study sample 2, "cannula group")

Figure 1B shows PMA-induced superoxide anion production by macrophages with WST-1 reduction scores plotted against increasing concentrations of PBMCs. WST-1 reduction scores differed significantly over PBMC concentrations ($F(3.7/14.9) = 37.14$, $p < .001$, $n = 5$).

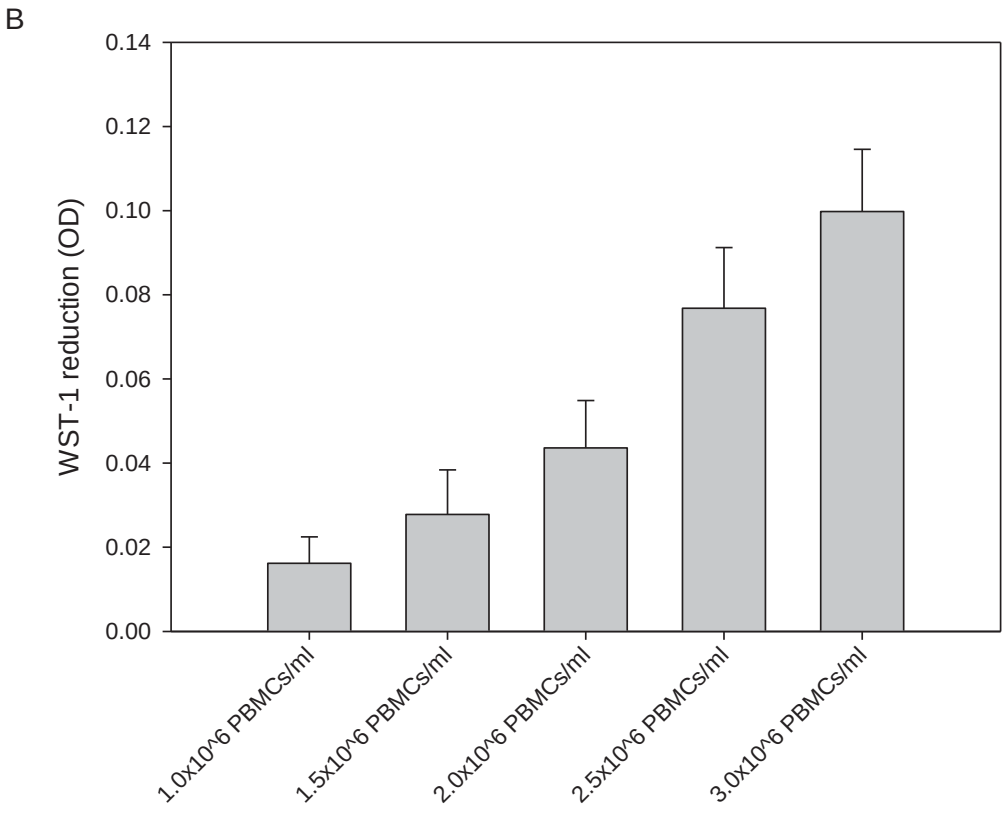

Figure 1B. WST-1 reduction scores with increasing concentrations of PBMCs.

Pilot data. Values are given as mean ± *SEM*. Figure 1B depicts PMA-induced superoxide anion production by macrophages with WST-1 reduction scores plotted against increasing concentrations of peripheral blood mononuclear cells (PBMCs).

3.2.2 Assay reliability (study sample 2, "cannula group")

Reliability assessment based on inter-assay variability of blood samples split before Ficoll purification was 9.77 % (± 6.91 SD, range = 0.00 – 21.65 %, *n* =23). Inter-assay variability of blood samples split after Ficoll purification was 10.92 % (± 6.55 SD, range = 0.95 – 21.93 %, *n* = 23).

3.2.3 Assay validation by comparison with reference method

3.2.3.1 Validation of the WST-1 macrophage assay by cytochrome c (study sample 2, "cannula group")

Higher cytochrome c reduction scores correlated significantly with higher WST-1 reduction scores (r = .57; p = .026; n = 15; Figure 2A).

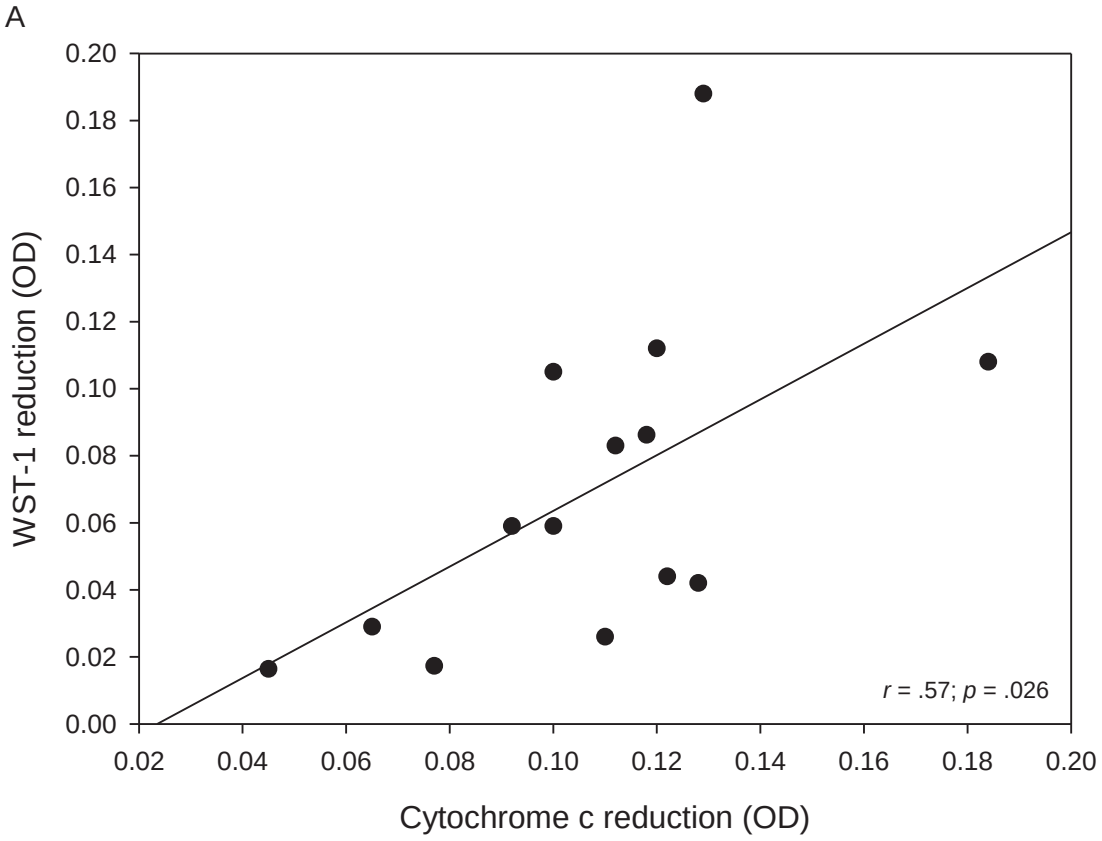

Figure 2A. Correlation between cytochrome c reduction scores and WST-1 reduction scores.

Validity. The scatter plot depicted in Figure 2A shows reduced cytochrome c and WST-1 reduction scores (cannula group).

3.2.3.2 Validation of macrophage superoxide anion production by generation of electrical current (study sample 3, "catheter group")

We observed current generation in HMDM of 21 participants (C_{Max} = 0.10 ± 0.01 ìA, range = 0.05-0.24 ìA; C_{Sum} = 284.12 ± 34.83 ìA, range = 139.01-765.77 ìA). Higher WST-1 reduction scores correlated significantly with higher current generation (r (WST-1 reduction / C_{Max}) = .48; p = .028; Figure 2B); r (WST-1 reduction / C_{Sum}) = .47; p = .033; Figure 2C). Exclusion

of two outlier subjects improved these positive associations (*r* (WST-1 reduction / C_{Max}) = .50; *p* = .030); *r* (WST-1 reduction / C_{Sum}) = .52; *p* = .024).

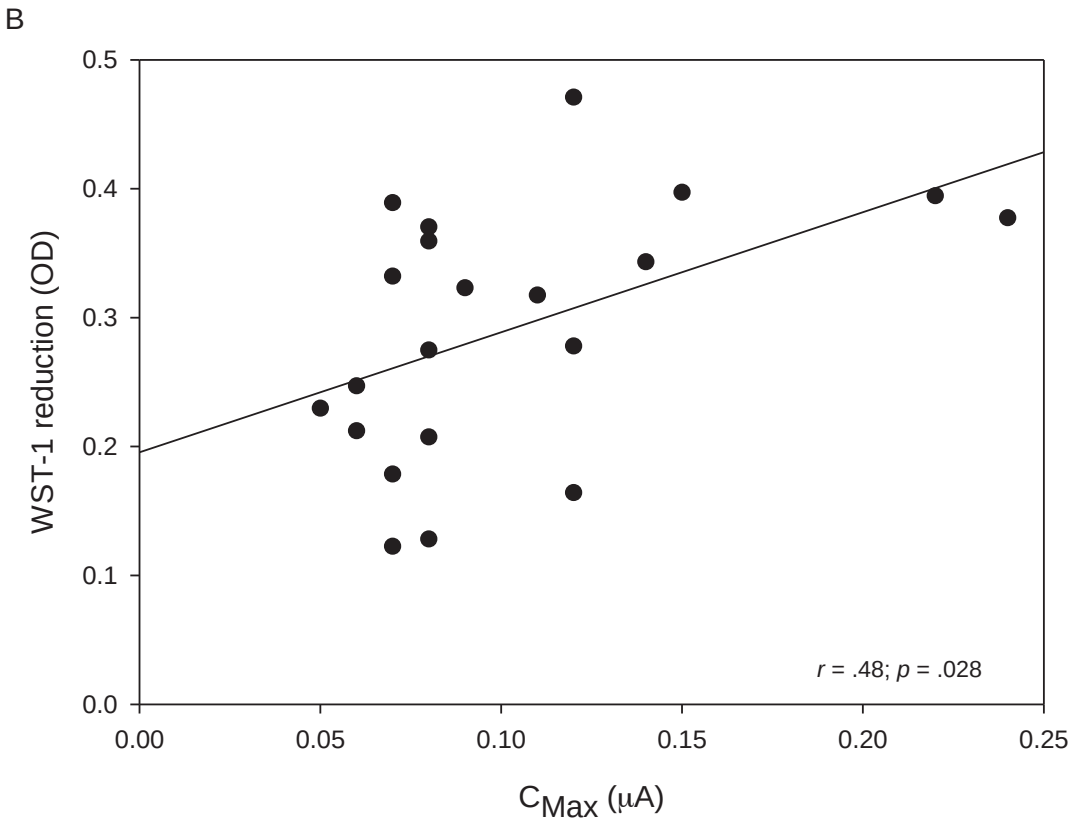

Figure 2B Correlation between WST-1 reduction scores and maximum current generation Validity. The scatter plot depicted in Figure 2B depicts the maximum current value registered (C_{Max}) and WST-1 reduction scores (catheter group).

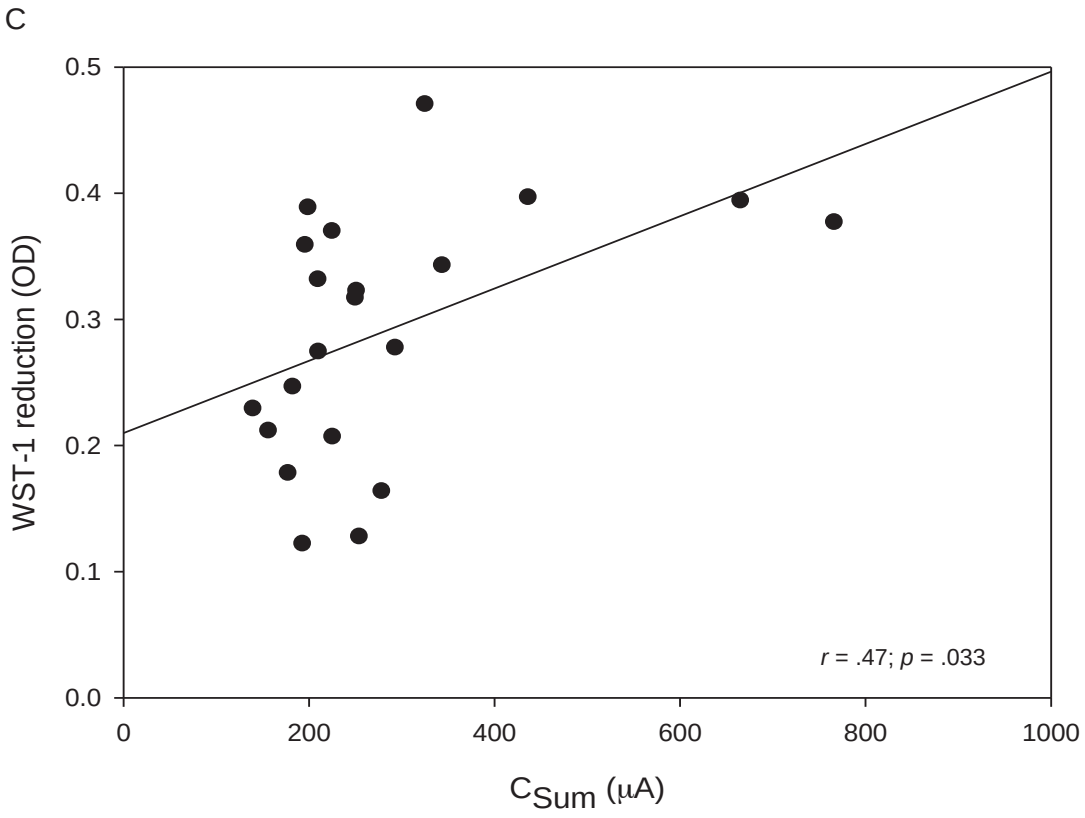

Figure 2C. Correlation between WST-1 reduction scores and sum of all current generation Validity. The scatter plot depicted in Figure 2C depicts the sum of all current values (C_{Sum}) and WST-1 reduction scores (catheter group).

3.2.4.2 Catheter group (study sample 3)

In the catheter group (n = 24), higher BDI scores correlated significantly with lower total WST-reduction scores measured from blood sampling obtained two hours after catheter insertion (r (BDI / total WST-1 reduction) = -.54, p = .010). Age and BMI were controlled. Without controlling for covariates results become of borderline significance (r (BDI / total WST-1 reduction) = -.35, p = .098).

4 DISCUSSION

In this study, we implemented and validated an *in vitro* method, that allows the investigation of NADPH oxidase-mediated microbicidal potential from *ex vivo* isolated HMDM.

In a pilot study, we verified applicability of the cell-line tested *in vitro* method for *ex vivo* isolated HMDM. Similar to THP-1 cells and independent of the blood sampling procedure, *ex vivo* isolated human monocytes / macrophages exposed to both differentiation and activation

agents produced the greatest amounts of superoxide anions compared to cells treated with either differentiating or activating stimuli alone. Application of the WST-1 macrophage assay evoked significant PMA-induced superoxide anion responses by HMDM in all participants of study sample 2 (cannula group) and study sample 3 (catheter group). These findings indicate that the *in vitro* method described for THP-1 cells is applicable to *ex vivo* isolated HMDM. We further showed that higher depressive symptoms and higher chronic stress were associated with higher WST-1 reduction scores ($r \geq .35$) in the cannula group. These data indicate that the WST-1 macrophage assay has utility for studies on effects of sustained psychological states on macrophage activity. Notably, the direction of these associations could be confirmed in men and women separately (data not shown).

Interestingly, comparable associations with psychological factors were obtained when the total WST-1 reduction scores were corrected for macrophage number per well (corrected WST-1 reduction scores). This suggests that determination of total WST-1 reduction scores seems to allow for adequate assessment of microbicidal potential per macrophage. However, it should be kept in mind that not the total WST-1 reduction scores, but rather the corrected WST-1 reduction scores reflect microbicidal potential per macrophage. Also, total WST-1 reduction scores reflect the microbicidal potential based on PBMC counts, i.e. the microbicidal potential of those macrophages differentiated from 2.5×10^6 PBMCs.

In contrast to the cannula group where positive associations between WST-1 reduction and depressive symptom severity were found, higher depressive symptom severity was associated with lower levels of WST-1 reduction ($r = -.54$) in the catheter group. This opposite finding not only corroborates the data above indicating assay sensitivity to psychological factors, but also provides first indication that depending on the activation status of the macrophages (i.e. basal activity of *in vivo* unstimulated cells or reactive to *in vivo* stimulation by catheter-induced wound-application), psychological factors can be associated in a different way with microbicidal potential of macrophages. Given this reasoning, the observed opposite association with depressive symptoms are not contradictory but rather present different phenomena. Thus, basal macrophage activity might be higher with increasing depression scores whereas reactive macrophage activity at the same time seems to be blunted. A speculative explanation for the observed opposite association with depressive symptoms may relate to exhaustion of this inflammatory macrophage type (i.e. decreased reactivity to *in vivo* stimulation by wound-induction) due to chronically elevated basal activation. But it also

cannot be ruled out that this observation relates to a potentially altered cell differentiation processes following wound-induction with depressive symptoms.

Furthermore, in a small subsample of study sample 2 (cannula group) we investigated PBMC-number dependent WST-1 reduction and found an increase in superoxide anion production with increasing PBMC concentrations in the range between 1.0 – 3.0 x 10^6 PBMCs. However, the superoxide anion production of 1.0 x 10^6 PBMCs was quite low suggesting that the minimum cell concentration of PBMCs needed to induce measureable PMA-induced superoxide anion release by HMDM after blood sampling by short-term cannula insertion ranges between 1.0 and 1.5 x 10^6 PBMCs. Higher PBMC cell numbers than this minimum concentration are therefore recommended to allow for optimal detection. In a larger subsample of study sample 2 (cannula group) we also tested assay reliability by using two different indicators of inter-assay variability. Inter-assay variabilities of the WST-1 macrophage assay were ≤ 10.92 % suggesting that the assay is sufficiently robust for quantitative research. Furthermore, we evaluated the validity of the assay by comparing WST-1 reduction scores with reference methods and found that greater WST-1 reduction was associated with greater cytochrome c reduction (cannula group) or current generation (catheter group), respectively. The observed moderate heights of the correlation coefficients in our study (r (WST-1 reduction / cytochrome c reduction) = .57; r (WST-1 reduction / C_{Max}) = .48; r (WST-1 reduction / C_{Sum}) = .47) suggest that the WST-1 macrophage assay provides a valid assessment of superoxide anion production by HMDM – especially when considering that current production in the fuel cell only partially originates from superoxide anions produced by HMDM, while the level of WST-1 reduction is almost completely based on HMDM superoxide anion release.

There are several potential implications for the implementation of the WST-1 macrophage assay. Thus far in the study of psychosomatic or psychobiological research, there has been a lack not only of methods for investigating microbicidal potential of human macrophages but also of literature regarding associations to longer-lasting psychological states or measures. However, elucidating the relationship between psychological factors and microbicidal potential of macrophages may contribute to a better understanding of the biological mechanisms linking psychological risk factors with adverse health consequences (e.g. increased susceptibility to infectious diseases, impaired wound healing, or coronary heart disease) where activated macrophages and thus peripheral immune cell activity play a major

role (Gouin & Kiecolt-Glaser, 2011; Mahdavian Delavary et al., 2011; Ross, 1999; Rozanski et al., 2005;). Moreover, several studies have suggested altered inflammatory activity and disturbed neuroendocrine-immune interaction in depression. Notwithstanding the fact that the clinical significance of the observed association between depressive symptoms and macrophage activity in our study remains unclear, it might be worthwhile to further investigate the role of macrophage activity in the interface between inflammation, neuroendocrine-immune interaction, and depression. The WST-1 macrophage assay may facilitate future research in this field.

Based on our implementation and validation data, we recommend the following for the use of the WST-1 macrophage assay: First, in order to investigate effects of potentially influencing factors on basal activity of M1 macrophages blood samples should be taken by means of short-term cannula insertion. This is because our data indicate that an indwelling venous catheter (at least if inserted for a longer period of time) seems to function as an open wound and to pre-activate circulating monocytes as precursors of later M1 macrophages *in vivo*. Second, although our data provide first indications that both total and cell-number corrected WST-1 reduction scores provide similar results in terms of associations with psychological factors we abstain from recommending to interpret total WST-1 reduction as an indicator of microbicidal potential per macrophage. Third, our data show acceptable inter-assay variability, which suggests that single measurements of WST-1 reduction provide acceptable results. Nevertheless it might be methodologically stronger to perform the assay in duplicates.

The present study has several strengths. First, it is the first study of tissue-based immune cell activity for use in psychosomatic research. Second, in comparison with other immunological methods or cell functionality assays, the *in vitro* method implemented in this study requires no expensive laboratory equipment or reagents, nor does it involve complicated procedures. Thus, we introduce a method that is also performable in smaller or less well equipped laboratories. Third, we implemented and validated the WST-1 macrophage assay for two commonly used blood sampling procedures, i.e. short-term cannula insertion and long-term catheter insertion, and in both women and men. The present study also has limitations. First, we did not systematically examine the association of macrophage microbicidal potential or ROS production with macrophage microbicidal activity. Although the important role of ROS in microbicidal activity of human mononuclear phagocytes is well established (de Oliveira-Junior et al., 2011), the relative importance of ROS in the microbicidal activity of

mononuclear phagocytes varies depending on the target pathogen (Cohen et al., 1981; Levitz & Diamond, 1985; Murray et al., 1985; Vazquez-Torres & Balish, 1997). Future research should examine the association of macrophage microbicidal potential with macrophage microbicidal activity. Second, we only implemented the macrophage assay using WST-1 reduction as detection method for macrophage superoxide anion production but not using other methods for superoxide anion detection, such as chemiluminescence- or flow cytometry-based methods. Further studies are needed to address this. Third, although our study provides first indications that depressive symptom severity and chronic stress affect macrophage microbicidal potential, we did not examine these associations systematically. Large-scale studies or studies involving depressive patients are needed to systematically address this (e.g. by comparing macrophage microbicidal potential between psychiatric patients and controls). A final limitation refers to the fact that no exclusion criteria were applied in the cannula group. While this procedure results in a high amount of generalizability on the one hand, we cannot rule out that results obtained from the cannula group or differential results between cannula vs. catheter group are biased by gender differences or potential unknown confounders on the other hand.

In summary, we present a successful *in vitro* method to determine peripheral immune cell activity. This can be accomplished by investigation of macrophage activation using a valid procedure of determining the NADPH oxidase-mediated microbicidal potential of *ex vivo* isolated HMDM. The present results indicate that this method is of interest to psychosomatic or psychobiological research as it allows to test for associations of psychological factors with peripheral immune cell activity. Additional research is needed to test the effects of acute mental stress responses and psychological constructs other than chronic stress or depressive symptoms. Future studies may also elucidate the effects of different assay modulation procedures such as variations in concentrations of stimulating agents and how such methods affect the observed associations between psychological factors and immune activation.

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SUPPLEMENTAL MATERIAL

Supplemental Digital Content 1

Determination of assay reliability

Assay reliability was tested by using two different measures of inter-assay variability. First, each blood sample was split into half rendering blood samples A and B of the same initial blood sample. Then, the WST-1 macrophage assay was started independently in both blood sample A and blood sample B. Second, after Ficoll density gradient purification and re-suspension of isolated PBMCs in RPMI medium at a density of 2.5 x 10^6 PBMCs / ml RPMI, the PBMC suspension of blood sample A was pipetted into two wells rendering the duplicate samples A1 and A2 before continuing with the WST-1 macrophage assay. Inter-assay variability was thus determined by comparing the amount of reduced WST-1 (1), in sample A1 and sample A2 assessing reliability after Ficoll purification procedure, as well as (2) in sample B and the mean of sample A1 and sample A2 assessing reliability before Ficoll purification procedure.

Supplemental Digital Content 2

Cytochrome c assay

In order to determine superoxide anion production by HMDM via cytochrome c reduction, cell preparation was performed exactly as described in section 2.3.1 and section 2.3.2. Then, the resulting macrophage monolayer was overlaid with 1 ml HBSS. Subsequently, 2 µl cytochrome c, 0.5 µl LPS, 2 µl IFN-γ, 2 µl TNF-α, and 0,5 µl PMA were added, resulting in a final concentration of 33 µM cytochrome c, 300 ng/ml LPS, 20 ng/ml IFN-γ, 20 ng/ml TNF-α, and 50 nM PMA. This was followed by an incubation period of 4 hours at 37°C and 5% CO_2. Then, the supernatant was removed and used to determine cytochrome c reduction by reading the OD with a spectrophotometer (Synergy HT, BioTek, Synergy HT, BioTek, Luzern, Switzerland) at 550 nm against water as blank as reduced cytochrome c shows increased absorbance at 550 nm. Increases in absorbance are proportional to the amount of cytochrome c reduced or superoxide anions generated by HMDM, respectively.

Supplemental Digital Content 3

Operation of the biofuel cell

The biofuel cell was constructed and operated as described by Sakai and colleagues (2009). Briefly, 2.5 x 10^6 PBMCs/ml were isolated as described in Section 2.3.1. Then, PBMC suspension aliquots of 3 ml were seeded on a gold electrode in custom-made incubation containers. For cell differentiation, we added 1.5 µl LPS, 6 µl IFN-γ, and 6 µl TNF-α resulting in equivalent final concentrations as for the WST-1 macrophage assay (300 ng/ml LPS, 20 ng/ml IFN-γ, and 20 ng/ml TNF-α; see Section 2.3.2). During a 48h incubation period at 37°C and 5% CO_2, HMDM became adherent to the electrode. After incubation, the electrode with HMDM was transferred to the anode compartment of a custom-made two-compartment biofuel cell. While the anode compartment was filled with 6 ml HBSS containing 300 ng/ml LPS, 20 ng/ml IFN-γ, and 20 ng/ml TNF-α, the fluid in the cathode compartment was HBSS with 0.1M potassium ferricyanide. For current generation, we added 3 µl PMA to the anode compartment corresponding to a concentration of 50 nM PMA. The PMA-induced current (µ Ampere, µA) was calculated from the voltage drop measured across a resistor with a digital multimeter (2000, Keithley Instruments Inc.). Current production was recorded continuously for four hours.

Supplemental Digital Content 4

Cell-counting method

Our WST-1 assay principle is based on monocytes included in 2.5 x 10^6 PBMCs that we stimulate by incubation with different agents to differentiate into macrophages. Our monocyte isolation method is a well-established procedure to yield monocyte cultures of high purity from PBMCs (Geng & Hansson, 1992; Mach et al., 1997; Pawlowski et al., 1983; Pawlowski et al., 1985; Selvan et al., 1997). Given the subsequent 48 h incubation time it can thus be assumed that resulting adherent cells are monocyte-derived macrophages. However, while WST-1 reduction scores are therefore unlikely to result from activation of cells others than macrophages, possible differences in WST-reduction scores may relate to differences in the number of macrophages derived from that same number of PBMCs. Notably, the same number of PBMCs may result in different numbers of adherent monocyte-derived macrophages. Consequently, we aimed to determine the number of adherent cells per well as an indicator of the final macrophage number per well. We corrected WST-1 reduction score

per 2.5 x 10^6 PBMCs (i.e. total WST-1 reduction scores) by post-hoc counted macrophage numbers to obtain a second measure of WST-1 reduction, i.e. macrophage number corrected WST-1 reduction score (corrected WST-1 reduction score).

In order to post-hoc determine the number of adherent macrophages per well we stained cells by applying a staining set and subsequently counted the stained cells. Staining was performed using "Diff-Quick Staining Set", a staining set that yields results comparable to the Pappenheim technique (i.e. the cytoplasm of monocytes / macrophages is stained sky blue and nuclei are stained violet). The staining procedure was as follows: After performing the WST-1 assay, the adherent macrophage layer was washed three times with 1 ml of warm (25°C) 0.01M PBS. Next, the adherent macrophage layer was overlaid with "Diff-Quick-Fix" solution for 30 seconds. After removing the "Diff-Quick-Fix" solution, the macrophage layer was overlaid with "Diff-Quick II" solution. After 30 seconds, the Diff-Quick II" solution was removed and the macrophage layer was overlaid with "Diff-Quick I" solution, again for 30 seconds. Then, "Diff-Quick I" was removed and the cell layer was washed three times with 1 ml of distilled water to remove excess stain. To count the number of adherent macrophages per well, stained cells were 40 times magnified and digitally photographed using a microscope with integrated camera (Nikon Eclipse TS1000, Nikon Industries, Egg, Switzerland). Stained cell per well were then counted from digital photographs by using Fiji ImageJ open source software (Schindelin et al., 2012). The number of adherent cells was determined in the first 30 participants of study sample 2 (15 women, 15 men).

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A3: MACROPHAGE SUPEROXIDE ANION PRODUCTION IN ESSENTIAL HYPERTENSION: ASSOCIATIONS WITH BIOLOGICAL AND PSYCHOLOGICAL CARDIOVASCULAR RISK FACTORS

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Abstract

Objective: Essential hypertension is an important risk factor for coronary artery disease and its underlying process atherosclerosis but involved mechanisms are not fully understood. Both macrophages and superoxide anions have been proposed to play a major role in the pathogenesis of atherosclerosis. In the present study we investigated whether macrophages of individuals with hypertension show higher nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-derived superoxide anion production compared to normotensives. Furthermore, we examined associations between macrophage superoxide anion production and the psychological factors depression and chronic stress independent from hypertension status.

Methods: We studied 30 hypertensive (M: 48.7 ± 2.4 years) and 30 age-matched normotensive men (M: 48.6 ± 2.4 years). We assessed macrophage superoxide anion production using the WST-1 assay. The assay bases on the chemical reduction of the cell-impermeative tetrazolium salt WST-1 by superoxide anions that are produced by activated human *ex vivo* isolated monocyte-derived macrophages. We further evaluated whether chronic stress or depressive symptom severity were associated with macrophage superoxide anion production. All analyses were adjusted for potential confounders.

Results: Individuals with hypertension showed higher superoxide anion production compared to normotensives ($F(1,58) = 11.56$, $p = .001$). Complementary analyses using mean arterial blood pressure (MAP) as a continuous measure revealed that higher MAP correlated significantly with higher WST-1 reduction ($ß = .38$, $p = .003$, $\Delta R^2 = .145$). These results remained significant when controlling for potential confounding influences. Chronic stress ($ß = .24$, $p = .067$, $\Delta R^2 = .053$) but not depression ($p = .24$) independently related to marginally higher WST-1 reduction scores.

Conclusions: Our results indicate higher macrophage superoxide anion production in individuals with hypertension compared to normotensives. This may suggest a mechanism underlying cardiovascular risk with hypertension.

1 INTRODUCION

Essential hypertension is a major risk factor for coronary artery disease (CAD) and its underlying process atherosclerosis (Ezzati et al., 2002; Frohlich et al., 1992; Kearney et al., 2005; Libby et al., 2011). However, the mechanisms that link hypertension with an increased risk of atherosclerosis are not fully understood (Alexander, 1995; Li & Chen, 2004).

The innate immune system plays a paramount role in initiation and progression of the inflammatory process in atherosclerosis, whereby monocytes and macrophages are key cells in this process (Ghattas et al., 2013; Linton & Fazio, 2003; Moore & Tabas, 2011; Ross, 1999). One of the earliest events in atherosclerosis is the recruitment of monocytes into the intima as the inner layer of the arterial wall, where they mature into macrophages being important mediators of the innate immune response and of inflammation in atherosclerosis (Ghattas et al., Libby, 2002; Libby et al., 2002; Moore et al., 2013).

A key innate immune effector function of classically activated macrophages (or inflammatory M1 macrophages, respectively) is microbicidal activity, i.e. the killing of microbes (Hunter et al., 2009; Martinez et al., 2008; Mosser & Edwards, 2008). Microbicidal effectiveness of human macrophages largely hinges on their production of reactive oxygen species (ROS; De Oliveira-Junior et al., 2011; Halliwell, 2006). ROS production in turn derives from the activated multisubunit enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase located in the phagolysosomal and plasma membrane of macrophages. Once activated, NADPH oxidase transfers electrons from NADPH in the cytosol to extracellular or intraphagolysosomal oxygen molecules. These oxygen molecules are then chemically reduced to highly reactive superoxide anions (O_2^-) and other ROS subtypes (Cathcart, 2004; De Oliveira-Junior et al., 2011). Particularly, superoxide anions are of major importance for the microbicidal activity of macrophages in host defence (Dale et al., 2008; Mosser & Edwards, 2008; Nathan & Shiloh, 2000).

Increasing evidence suggests that NADPH oxidase and the resulting production of superoxide anions are likely to play a critical role in the pathogenesis of atherosclerosis. For instance, NADPH oxidase-derived superoxide anions can induce low-density lipoprotein oxidation (Bey & Cathcart, 2000; Cathcart, 2004; Cathcart et al., 1985; Griendling et al., 2000; Vendrov et al., 2007), an important cause of endothelial dysfunction as an initial step in atherosclerosis (Libby et al., 2011; Ross, 1999). Indeed, NADPH oxidase-deficient mice developed significantly less atherosclerosis as assessed by quantifying atherosclerotic lesion sizes (Barry-Lane et al., 2001). Furthermore, in mice the extent of atherosclerosis correlated with

higher aortic superoxide anion production (Vendrov et al., 2007). Similarly, a study in human coronary arteries revealed that NADPH oxidase-mediated superoxide anion production was highest in the macrophage-rich shoulder regions of the plaque and higher superoxide anion production correlated with higher severity of atherosclerosis (Sorescu et al., 2002).

To date, NADPH oxidase-derived superoxide anion production by inflammatory macrophages as cells present in atherosclerotic lesions has not yet been investigated in essential hypertension. Also, a potential role of superoxide anion production by inflammatory macrophages in mediation of atherosclerotic risk in hypertension is unclear. So far, two studies investigated NADPH oxidase-derived superoxide anion production in macrophage precursor cells, namely circulating peripheral blood mononuclear cells (PBMCs). So, Fortuño and colleagues reported a superoxide anion overproduction in circulating PBMCs among partially treated hypertensive men and women compared to normotensives (Fortuño et al., 2004). Furthermore, Watanabe and colleagues observed in individuals with hypertension a higher ROS formation by circulating mononuclear cells with increasing carotid intima-media thickness (IMT) as a vague index for atherosclerosis severity (Watanabe et al., 2006).

Here, we investigate for the first time whether individuals with hypertension differ from normotensives in their NADPH oxidase-derived superoxide anion production by inflammatory M1 macrophages. To account for a confounding influence by age (Ferrara et al., 1997), subject groups were matched for age. Given the atherosclerotic risk of hypertension in combination with the supposed role of inflammatory macrophages and superoxide anions in atherosclerosis, we hypothesized individuals with hypertension to show a higher macrophage superoxide anion production as compared to normotensives.

2 MATERIALS AND METHODS

2.1 Study participants

This study was part of a larger project assessing psychoneurobiological mechanisms in essential hypertension. The project was approved by the Ethics Committee of the State of Bern, Switzerland, and all participants provided written informed consent.

Between December 2012 and May 2014, we recruited by aid of the Swiss Red Cross of the State of Bern apparently healthy, nonsmoking, and medication-free hypertensive and age-matched normotensive men. In detail, members of our study team accompanied the mobile blood-donation unit of the Swiss Red Cross that routinely records blood pressure (BP) before blood donation. Male blood donors with elevated BP and expressing interest in participating

in the study were given the written study information asking for the following inclusion criteria: age 18 – 80 years, systolic BP ≥ 140 mmHg and/or diastolic BP ≥ 90 mmHg; nonsmoker; no acute or regular intake of medication; and no alcohol or illicit drug abuse. Next, we assessed whether interested blood donors were actually hypertensive or not (see below). Those identified as hypertensive were then screened by telephone interview using an extensive health questionnaire. Explicit exclusion criteria were: regular strenuous exercise, alcohol and illicit drug abuse, liver and renal diseases, chronic obstructive pulmonary disease, allergies and atopic diathesis, rheumatic diseases, human immunodeficiency virus, cancer, major psychiatric disorders, neurological diseases, and current infectious diseases. In addition, eligible hypertensive participants provided blood samples for the routine assessment of serum creatinine, calcium, sodium, and potassium to find potential cases with secondary hypertension. Furthermore, we measured HbA1c and LDL / HDL ratio in all participants. All participants eligible for our study were assessed for the macrophage activation assay as described below. In two out of 32 recruited hypertensive men, macrophage activation data could not be obtained due to assay problems. No eligible hypertensive participant was diagnosed with secondary hypertension post-hoc, so all were defined to have essential hypertension. Notably, calcium, sodium, and potassium could not be analyzed in 3 hypertensive participants and HbA1c and LDL/HDL ratio could not be assessed in 2 normotensive participants because of technical problems. For each of the 30 essential hypertensive participants with macrophage data we recruited an age-matched (± 4years) normotensive control on a case-by-case basis to balance with regard to potential confounding effects of age on superoxide anion production (Fulop et al., 2004), yielding a final study sample of 60 participants. Apart from hypertension-related criteria, controls had to meet the same inclusion and exclusion criteria as individuals with hypertension.

2.2 Assessment of essential hypertension

Following written instructions, each participant was required to measure BP on three separate days at home using sphygmomanometry (Omron M6; Omron Healthcare Europe B.V., Hoofdorp, Netherlands). Home BP measurements were obtained in a seated position after a 15-minute rest twice per day (once in the morning and once in the evening, rendering a total of 6 measurements for each participant) and the average BP was computed. Deviating from instructions, 2 normotensive and 7 hypertensive participants provided 6 BP measurements on 4 to 6 different days and 1 normotensive participant measured BP only once. Hypertension

was conservatively defined (both for home and study measurements) by the World Health Organisation (WHO) / Internation Society of Hypertension (ISH) definition; that is systolic BP ≥ 140 mmHg and / or diastolic BP ≥ 90 mmHg (Chalmers et al., 1999). Participants were conservatively classified as normotensive if home systolic BP was < 135 mmHg and home diastolic BP was < 85 mmHg according to recommendations for home BP measurements (Mancia et al., 2013). Due to technical problems, home BP was missing in 2 hypertensive and 2 normotensive participants who were alternatively classified based on their BP values recorded by the blood donation center. The classification of each participant as hypertensive or normotensive was verified by three additional seated study BP measurements after a 15-minute rest performed by trained personnel during the study session (see below). Six normotensive participants provided only two instead of three of those additional seated study BP measurements. Notably, for classification of normotension according to study BP measurements, we applied the regular WHO/ISH definition; that is systolic BP < 140 mmHg and diastolic BP < 90 mmHg. We calculated mean arterial blood pressure (MAP) from the study BP measurements by the formula (2/3mean diastolic BP) + (1/3mean systolic BP). Table 1 gives an overview of the BP measurement procedure and the different cutoff values (Figure 1) for the classification of hypertension and normotension in our study.

Table 1. Cutoff values for the classification of hypertension and normotension

	Hypertension	Normotension
Blood pressure		
Home SBP (mmHg)	≥ 140 mmHg	< 135 mmHg
Home DBP (mmHg)	≥ 90 mmHg	< 85 mmHg
Study SBP (mmHg)	≥ 140 mmHg	< 140 mmHg
Study DBP (mmHg)	≥ 90 mmHg	< 90 mmHg

Notes: Home SBP = systolic blood pressure from home measurements; home DBP = diastolic blood pressure from home measurements; study SBP = systolic blood pressure from study measurements; study DBP = diastolic blood pressure from study measurements

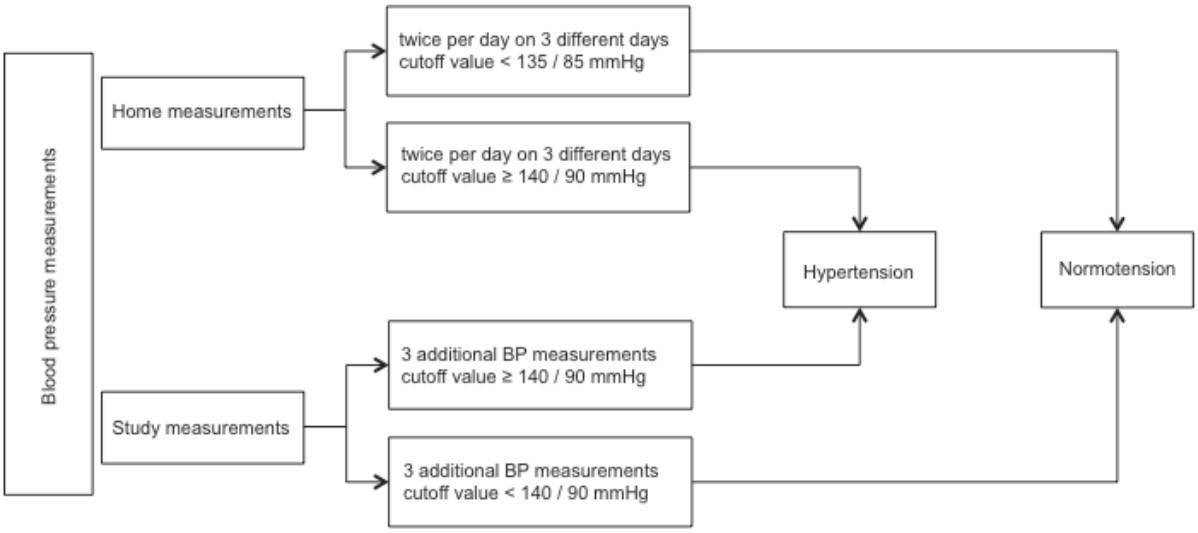

Figure 1. Procedure for the classification of hypertension and normotension

Procedure of blood pressure (BP) measurements for the classification of hypertension and normotension.

2.3 Procedure

All participants abstained from caffeine and alcohol consumption for 24 h and consumed a semi-standardized breakfast following written instructions prior to arrival at the lab at 8:00 h. Then questionnaires were administered. Blood was collected by short-term cannula insertion (see Kuebler et al., 2013) for the assessment of superoxide anion production at 11:30 h, i.e., after a fasting for 3.5 h since arrival. BP was assessed by means of sphygmomanometry (Omron M6; Omron Healthcare Europe B.V., Hoofdorp, Netherlands) 3 h and 2.5 h before and 10 min after blood sampling. In one participant BP measurement was taken only once 10 min after blood sampling due to technical problems.

2.4 Macrophage activation assessment

2.4.1 Reagents and chemicals

We used the following reagents: Ficoll-Paque PLUS (Ficoll; no. 17-1440-02; GE Healthcare, Uppsala, Sweden), 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)2H-tetrazolium (WST-1; no. 150849-52-8; Dojindo Laboratories, Kumamoto, Japan), interferon-γ (IFN-γ; no. PHC4031; Invitrogen, Basel, Switzerland), tumor necrosis factor-α (TNF-α; no. PHC3016; Invitrogen, Basel, Switzerland), Hank's balanced salt solution without phenol red (HBSS; no. 14025050; Invitrogen, Basel, Switzerland), fetal bovine serum (FBS; no. 10270-106;

Invitrogen, Basel, Switzerland), lipopolysaccharide (LPS; no. L6529; Sigma-Aldrich, Buchs, Switzerland), phosphate-buffered saline (PBS; no. P5368; Sigma-Aldrich, Buchs, Switzerland), phorbol 12-myristate 13-acetate (PMA; no. P8139; Sigma-Aldrich, Buchs, Switzerland), RPMI 1640 medium with glutamax (RPMI 1640; no. W9925E; Fisher Scientific, Wohlen, Switzerland), and Diff-Quick Staining Set (Medical Solutions GmbH, Hünenberg, Switzerland).

2.4.2 WST-1 assay

We assessed superoxide anion production of *ex vivo* isolated human monocyte-derived M1 macrophages (HMDM) based on our recently validated *in vitro* WST-1 assay (Kuebler et al., 2013). The assay principle is based on the chemical reduction of the cell-impermeative tetrazolium salt WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt) by superoxide anions that are produced by PMA-activated HMDM, which represent the classically activated M1 macrophages (Martinez et al., 2008). The assay procedure is described in detail in the provided Supplemental Material. Higher ODs, as obtained in absorbance reading, are associated with higher amounts of WST-1 reduction and thus of superoxide anions generated by HMDM. In 3 hypertensive and 3 normotensive men the assays were not performed in duplicates due to low PBMC numbers.

2.4.2.1 Cell number correction of WST-1 reduction scores

To rule out that possible differences in WST-reduction scores may relate to differences in the number of macrophages derived from 3.0 x 10^6/ml PBMCs, we followed previous methods (Kuebler et al., 2013) and determined the number of adherent cells per well as an indicator of the final macrophage number per well and calculated WST-1 reduction scores per 10’000 macrophages (corrected WST-1 reduction score). The procedure to correct for the number of cells is detailed in the provided Supplemental Material.

2.5 Psychological Assessment

The potential association of depressive symptom severity and chronic stress with superoxide anion production (Kuebler et al., 2013) was examined using regression analyses.

2.5.1 Depressive Symptom Severity

Depressive symptom severity was measured with the validated German version (Hautzinger et al., 2006) of the 21-item Beck Depression Inventory – Second Edition (BDI-II; Beck et al., 1996), where scores of 11 or higher indicate possible clinical depression. The BDI-II measures a somatic and a cognitive-affective dimension of depression and assesses the frequency and / or severity of symptoms related to sadness, feelings of guilt, perceptions of self-worth, suicidal ideation, and changes in appetite and body weight, among other characteristics. Items are rated on a 4-point Likert scale ranging from 0 (symptom not present) to 3 (symptom very much present) that add to a total BDI score ranging from 0 to 63. Higher scores indicate higher depressive symptom severity. Cronbach's alpha of the BDI total score was .89 in our sample. In analyses controlling for a potential influence of BDI, missing BDI data of one hypertensive and one normotensive participant were estimated using the expectation-maximization (EM) algorithm (Hippel, 2004; Moon, 1996).

2.5.2 Chronic Stress

To assess chronic stress we used the 12-item Chronic Stress Screening Scale (CSSS; Schulz et al., 2004). The CSSS includes questions about frequency of experiencing work overload (four items), worries (four items), lack of social recognition (two items), excessive demands at work (1 item) and social overload (1 item). Items have a 5-point rating format reflecting stress frequency (1 = "never" to 5 = "very often"). Possible scores range from 12 to 60 with higher scores meaning greater levels of chronic stress. Cronbach's alpha of the CSSS total score was .91 in our sample. Missing CSSS data of 1 normotensive and 3 hypertensive participants were estimated using the EM algorithm (Hippel, 2004; Moon, 1996) and results were used in analyses controlling for a potential influence of chronic stress.

2.6 Statistical analysis

Data were analyzed using SPSS (Version 20) statistical software package for Macintosh (IBM SPSS Statistics, NY, USA). All analyses were two-tailed, with the level of significance set at $p < .05$. Results are shown as mean ± SD.

Prior to statistical analyses, data were tested for normal distribution and homogeneity of variance using Kolmogorov-Smirnov and Levene's tests, respectively. All data were normally distributed within the two groups but homogeneity of variance of WST-1 reduction scores was not verified in the group comparisons. To calculate univariate analyses of variance

(ANOVAs) testing for group differences we therefore logarithmically transformed WST-1 reduction scores and homogeneity of variance was verified. For reasons of clarity we show original WST-1 reduction scores in all figures. Body mass index (BMI) was calculated as the ratio of weight in kilograms to height in square meters. WST-1 scores are presented with and without correction for macrophage numbers: "corrected WST-1 reduction" refers to WST-1 reduction scores per 10'000 macrophages.

We used ANOVAs to test for differences in the characteristics of the two groups and to test whether individuals with hypertension exhibited higher WST-1 reduction scores or higher corrected WST-1 reduction scores as compared to normotensives. To investigate linear associations and to take into account that our age-matching procedure may compromize independence of the two study groups, we calculated complementary analyses using multivariate linear regression models (enter method) with the continuous measure study MAP instead of the dichotomous (age-matched) group variable. We tested whether WST-1 reduction scores or corrected WST-1 reduction scores (dependent variables) were associated with study MAP, CSSS, or BDI (independent variables). In all WST-1 reduction analyses of the study (i.e. ANOVAs and regression analyses) we adjusted for traditional cardiovascular risk factors (i.e. BMI, LDL/HDL ratio, creatinine), for psychological factors (i.e. BDI and CSSS), and for the full set of these potential confounders. In linear regression models with MAP, instead of the age-matched group variable, we additionally adjusted for age. Control variables were selected a priori based on previous literature showing associations with immune activation or superoxide anion production of HMDM, respectively (Dorshkind et al., 2009; Kuebler et al., 2013; Wirtz et al., 2008).

3 RESULTS

3.1 Group characteristics

Hypertensive participants, as expected, presented with a higher systolic BP, a higher diastolic BP and a higher MAP as compared to normotensive participants. In addition, individuals with hypertension had a higher BMI and LDL/HDL ratio than normotensives. The two groups did not significantly differ in terms of age, BDI, CSSS, and HbA1c (see Table 2). Hypertensive participants had serum levels of creatinine, calcium, sodium, and potassium in the normal reference range, so supporting a diagnosis of essential hypertension. WST-1 reduction scores related to all BP measures but to none of the other group characteristic parameters. The unadjusted correlations for the full group are provided in the Supplemental Material.

Table 2. Group characteristics of hypertensive and normotensive participants

	Individuals with hypertension n = 30	Normotensives n = 30	*P*-value
Age (yrs)	48.7 ± 13.0 (21-74)	48.6 ± 13.8 (22-74)	.98
BMI (kg/m^2)	28.4 ± 3.5 (21.6-34.6)	24.9 ± 2.9 (19.8-30.9)	< .001
Blood pressure			
Home SBP (mmHg)	143.6 ± 7.8 (115.7-158.5)	120.3 ± 6.6 (105.2-129.3)	< .001
Home DBP (mmHg)	86.7 ± 10.0 (66.0-117.5)	70.8 ± 5.5 (60.0-80.8)	< .001
Study SBP (mmHg)	149.2 ± 9.5 (129.3-173.3)	122.4 ± 6.3 (108.7-137.3)	< .001
Study DBP (mmHg)	93.2 ± 9.5 (73.7-113.7)	76.8 ± 6.4 (58.3-85.0)	< .001
MAP (mmHg)	111.9 ± 8.9 (95.8-133.4)	92.0 ± 6.0 (75.3-99.5)	< .001
LDL/HDL ratio (µmol / L)	2.8 ± 0.8 (1.4-4.3)	2.2 ± 0.7 (1.1-4.4), n=28	.007
Creatinine (µmol / L)	79.9 ± 9.5 (66-100)		
Sodium (mmol / L)	140.4 ± 1.8 (137-144), n=27		
Calcium (mmol / L)	2.4 ± 0.1 (2.1-2.6), n=27		
Potassium (mmol / L)	4.2 ± 0.2 (3.9-4.7), n=27		
HbA1c (mmol/mol)	36.6 ± 3.2 (29-43)	36.1 ± 2.9 (30-41), n=28	.60
Chronic stress (CSSS)	11.9 ± 8.2 (1-35), n=27	13.0 ± 7.7 (0-26), n=29	.62

Depressive symptom severity (BDI)	3.52 ± 4.7 (0-18), n=29	4.59 ± 4.9 (0-19), n=29	.41

Notes: Values are means ± SD (range); BMI = body mass index; SBP = systolic blood pressure; DBP = diastolic blood pressure; MAP = mean arterial blood pressure, CSSS = Chronic Stress Screening Scale; BDI = Beck Depression Inventory; n = number of participants in case of missing data.

3.2 Group differences in WST-1 reduction scores

We observed that individuals with hypertension had significantly higher WST-1 reduction scores compared to normotensives, either without ($F(1,58) = 11.56$, $p = .001$, $Eta^2 = 0.17$, $f = 0.45$, Figure 2) or with correction for macrophage numbers ($F(1,58) = 6.98$, $p = .011$, $Eta^2 = 0.11$, $f = 0.35$). Controlling for BMI, LDL/HDL ratio, and creatinine (WST-1 reduction score: $F(1,53) = 13.40$, $p = .001$, $Eta^2 = 0.13$, $f = 0.38$; corrected WST-1 reduction score: $F(1,53) = 8.34$, $p = .006$, $Eta^2 = 0.10$, $f = 0.34$) or CSSS and BDI (WST-1 reduction score: $F(1,56) = 13.14$, $p = .001$, $Eta^2 = 0.19$, $f = 0.48$; corrected WST-1 reduction score: $F(1,56) = 8.31$, $p = .006$, $Eta^2 = 0.13$, $f = 0.38$) did not change results. The effect persisted when controlling for the full set of confounding variables (WST-1 reduction score: $p < .001$; corrected WST-1 reduction score: $p = .003$).

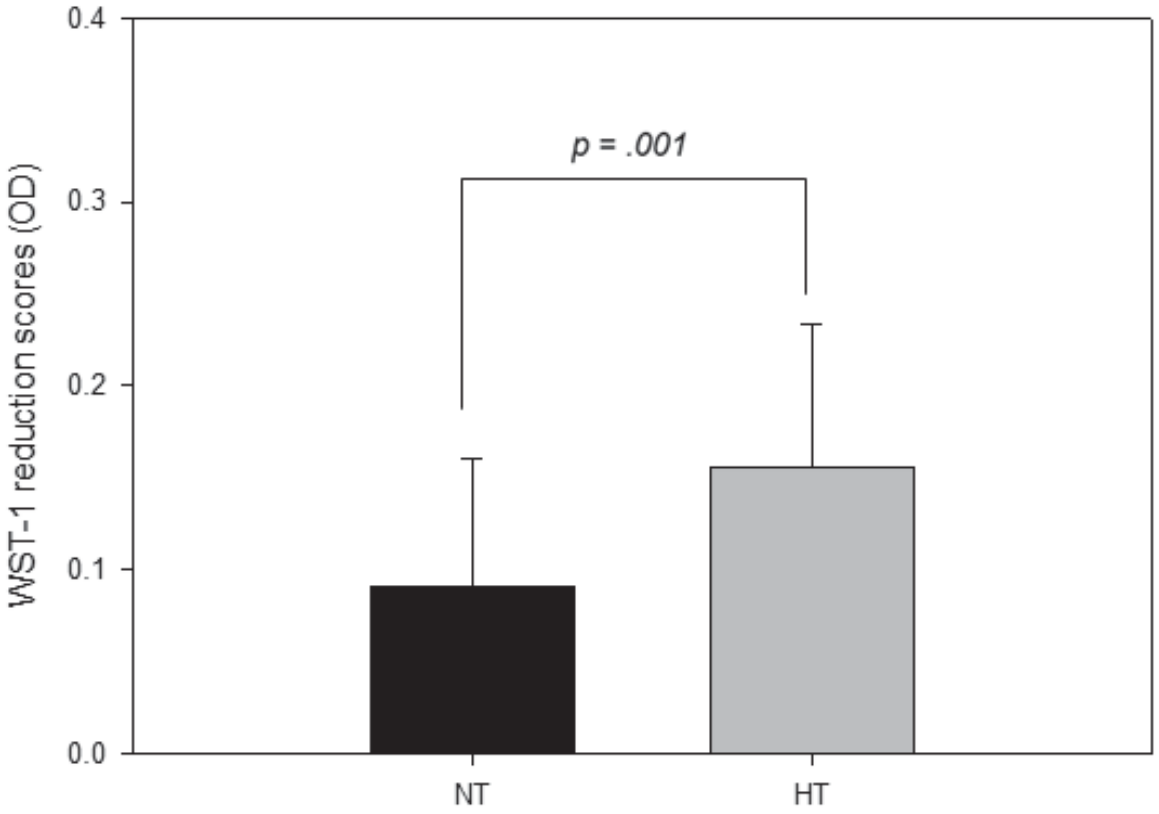

Figure 2. Group differences in WST-1 reduction scores

WST-1 reduction scores in hypertensives (HT) and normotensives (NT) men (mean ± SD). Hypertensive men had significantly higher WST-1 reduction scores than normotensive men ($p = .001$).

3.3 Association between WST-1 reduction scores and mean arterial blood pressure

Complementary analyses using study MAP as a continuous measure revealed that higher MAP correlated significantly with higher WST-1 reduction scores, either without ($\beta = .38$, $p = .003$, $\Delta R^2 = .145$, Figure 3) or with macrophage number correction ($\beta = .35$, $p = .006$, $\Delta R^2 = .124$). Results remained significant after controlling for age, BMI, LDL/HDL ratio, and creatinine (WST-1 reduction score: $\beta = .43$, $p = .007$, $\Delta R^2 = .124$; corrected WST-1 reduction score: $\beta = .43$, $p = .004$, $\Delta R^2 = .131$) or BDI and CSSS (WST-1 reduction score: $\beta = .40$, $p = .002$, $\Delta R^2 = .155$; corrected WST-1 reduction score: $\beta = .37$, $p = .004$, $\Delta R^2 = .133$). Controlling for age, BMI, LDL/HDL ratio, and creatinine in addition to BDI (WST-1 reduction score: $p = .006$; corrected WST-1 reduction score: $p = .004$) or CSSS (WST-1 reduction score: $p = .006$; corrected WST-1 reduction score: $p = .005$) did not significantly change results.

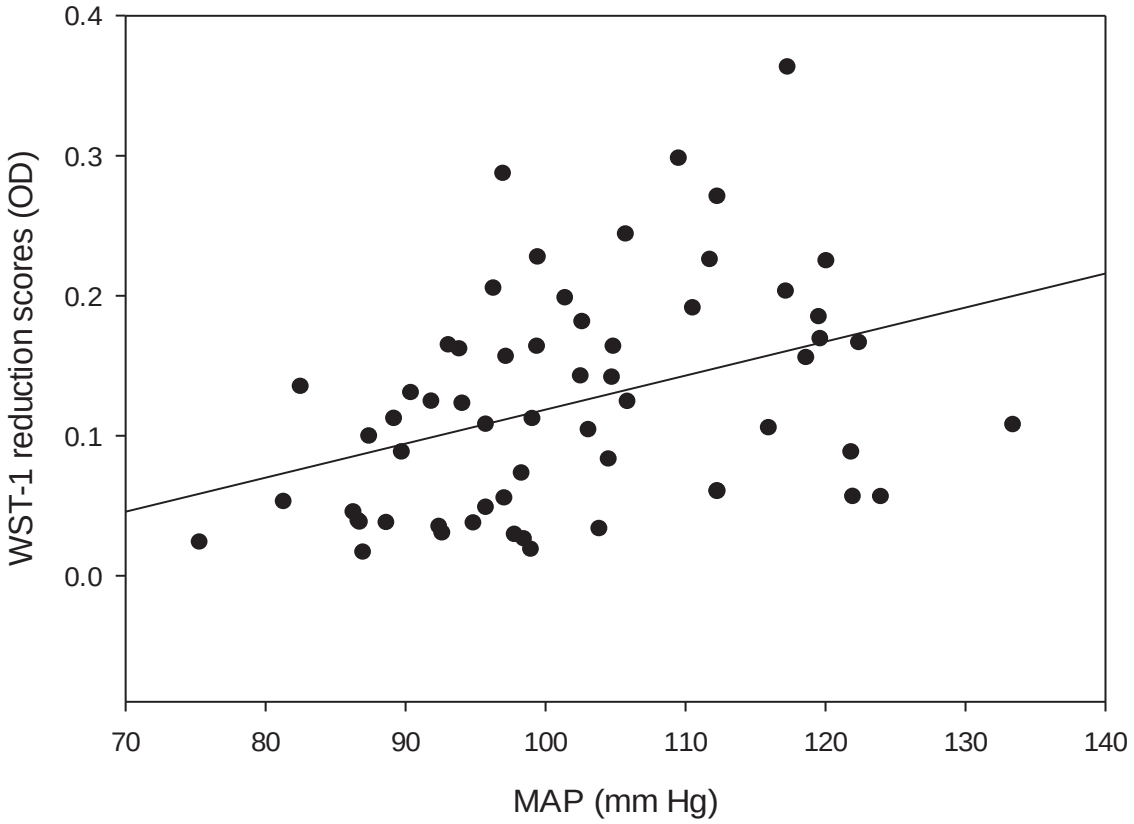

Figure 3. WST-1 reduction scores in relation to mean arterial blood pressure (MAP)
The scatter plot shows a linear positive relationship between mean arterial blood pressure (MAP) and WST-1 reduction scores ($ß$ = .38, p = .003).

3.3 Associations between WST-1 reduction scores, chronic stress, and depressive symptom severity

We further correlated WST-1 reduction scores with CSSS and BDI for the full group. CSSS (WST-1 reduction score: β = .22, p = .098, ΔR^2 = .047; corrected WST-1 reduction score: β = .23, p = .074, ΔR^2 = .054) but not BDI (WST-1 reduction score: β = .16, p = .236, ΔR^2 = .024; corrected WST-1 reduction score: β = .19, p = .148, ΔR^2 = .036) was marginally positively associated with WST-1 reduction scores. After controlling for age, BMI, MAP, LDL/HDL ratio, and creatinine both higher CSSS and higher BDI related to (marginally) higher corrected WST-1 reduction scores (CSSS: WST-1 reduction score: β = .24, p = .067, ΔR^2 = .053; corrected WST-1 reduction score: β = .28, p = .031, ΔR^2 = .074; BDI: WST-1 reduction score: β = .15, p = .238; corrected WST-1 reduction score: β = .23, p = .078, ΔR^2 = .051).

4 DISCUSSION

In this study, we investigated for the first time whether hypertensive men differ from age-matched normotensive men in terms of superoxide anion production by *ex vivo* isolated monocyte derived macrophages. In complementary analyses, we additionally examined the association between MAP as a continuous measure and superoxide anion production.

We found that our hypertensive participants showed higher WST-1 reduction scores of PMA-activated *ex vivo* isolated monocyte-derived macrophages than normotensive controls. In addition, higher MAP was independently associated with higher superoxide anion production. Moreover, chronic stress but not depressive symptom severity was marginally associated with higher macrophage superoxide anion production. Given the importance of superoxide anions for microbicidal activity (Dale et al., 2008; Mosser & Edwards, 2008; Nathan et al., 2000), our results suggest that macrophages of individuals with hypertension are characterized by an increased preparedness to kill microbes in reaction to stimulating agents. Importantly, we found this association to be linear, i.e. the preparedness to kill microbes rose with increasing MAP.

Our observation is in line with previous studies that also found increased NADPH oxidase-derived ROS production by circulating macrophage precursor cells in human hypertension (Fortuño et al., 2004; Watanabe et al., 2006). Our study extends the previous findings by pointing to tissue-based monocyte-derived inflammatory macrophages and thus cells known to be present in atherosclerotic lesions as the source of higher superoxide anion production in human hypertension. In individuals with hypertension higher ROS formation by circulating PBMCs including monocytes has been found to correlate with increasing IMT as a proxy measure of atherosclerosis severity (Watanabe et al., 2006). If this correlation also applies to monocyte-derived tissue-based inflammatory macrophages as key cells in atherosclerosis development and progression, our findings may suggest a mechanism underlying the cardiovascular risk with hypertension.

At present, we can only speculate about mechanisms underlying the observed higher phagocytic NADPH oxidase-derived superoxide anion production in hypertension. Several prospective studies in humans reported that inflammatory processes can be predictive for the development of hypertension (Engström et al., 2002; Niskanen et al., 2004; Sesso et al., 2003). Experimental animal studies confirmed this causal relationship (Ghattas et al., 2013; Harrison et al., 2011) and suggested that inflammatory mechanisms may prime macrophages to increased microbicidal preparedness (Gauss et al., 2007; Schroeder et al.,

2004). Moreover, it is conceivable that increased levels of oxidized LDL in individuals with hypertension (Forstegar et al., 2003) may trigger macrophages to produce greater amounts of superoxide anions in order to ingest oxidized LDL. This may promote vascular wall injuries with subsequent chronic inflammation that ultimately result in atherosclerotic events.

Therapeutic implications of our findings may relate to modulation of NADPH oxidase-derived superoxide anion production in the treatment of hypertension and prevention of atherosclerotic risk. A potential treatment option may include antioxidants, e.g. vitamin C, that inhibit NADPH oxidase-derived generation of ROS (Schiffrin, 2010; Touyz & Schiffrin, 2004; Yin et al., 2014). Furthermore, antihypertensive agents such as AT-II receptor antagonists are known to inhibit NADPH oxidase activity and thus decreasing the generation of ROS production (Yin et al., 2014).

Notably, our study was cross-sectional and hence we can not draw a conclusion on the direction of the hypertension-microbicidal activity-atherosclerosis-link. Prospective studies or randomized controlled trials are needed to address this important issue. The present study focused on apparently healthy, nonsmoking, and medication-free men and may therefore not be generalized to women or patients with other forms of cardiovascular disease. Also, although unlikely, we cannot completely rule out that some of the hypertensive participants may suffer from secondary hypertension. Moreover, we did not examine the association of superoxide anion production with macrophage microbicidal activity, e.g. in atherosclerotic lesions. Nevertheless, this is the first study that investigated superoxide anion production by *ex vivo* isolated HMDM in human hypertension using the reliable, and valid WST-1 assay. Our observation of increased superoxide anion production may provide new insights into mechanisms that underlie the increased risk of atherosclerosis and CAD in hypertension. Furthermore, the inclusion of apparently healthy, nonsmoking, and medication-free hypertensive men and age-matched normotensives reduced potential confounding factors, while controlling for selected other potential confounders. Nevertheless, we cannot rule out that potential confounders that we did not assess may have an impact on macrophage superoxide anion production and thus on the results reported above. Of note, in spite of missing data we consider the collected BP measurements to be reliable since missing data points resulted from problems other than participants' instruction adherence.

In sum, our data show that macrophage superoxide anion production is higher in hypertensive men compared to normotensives suggesting a mechanistic role in mediation of

cardiovascular risk in hypertension with potential implications for intervention strategies. Future studies are needed to confirm our findings and to further investigate the precise mechanisms underlying atherosclerotic risk in human hypertension.

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SUPPLEMENTAL MATERIAL

Supplemental Digital Content 1

WST-1 assay procedure

The WST-1 assay was performed based on Kuebler et al. (2013). In brief, 9 ml of blood were collected in EDTA-coated tubes (Sarstedt, Numbrecht, Germany), immediately layered on top of 10 ml Ficoll, and centrifuged for 20 min at 300 g and 20°C. After centrifugation, PBMCs were removed from the interface, washed twice in RPMI-1640 medium, counted with a hematologic analyzer (KX-21N; Sysmex Digitana AG), re-suspended to a final concentration of 3.0 x 10^6/ml with RPMI-1640 media supplemented with 10% FBS. Then, PBMC suspension aliquots of 1 ml were transferred to 24-well cell culture plates (no. 4609; Semadeni; Ostermundigen, Switzerland). After incubation for 2 hours at 37°C with 5% CO_2, the supernatant was discarded and the plate surface was rinsed five times with 1 ml of warm (25°C) 0.01 M PBS to remove non-adherent PBMCs, while monocytes remained adherent to the bottom of the plate.

The adherent monocyte layer was then diluted with 1 ml RPMI-1640 media supplemented with 10% FBS. Subsequently, we added IFN- γ, TNF- α, and LPS in a final concentration of 20 ng/ml IFN- γ, 20 ng/ml TNF- α, and 300 ng/ml LPS to promote differentiation of monocytes into inflammatory macrophages. After incubation for 44 h at 37°C with 5% CO_2, the supernatant was discarded and the adherent macrophage layer was washed twice with 1 ml of warm (25°C) 0.01 M PBS to remove traces of culture media and non adherent cells.

Next, the resulting macrophage monolayer (obtained as described above) was overlaid with 1 ml HBSS. Subsequently, IFN- γ, TNF- α, LPS, WST-1 and PMA were added, resulting in a final concentration of 20 ng/ml IFN- γ, 20 ng/ml TNF- α, 300 ng/ml LPS, 100 μM WST-1 and 50 nM PMA. After an incubation period of 4 hours at 37°C with 5% CO_2, the supernatant was removed and used to determine WST-1 reduction by reading the optical densities (ODs) with a spectrophotometer (Tecan infinite M1000, Tecan, Salzburg, Austria) at 450 nm against water as blank.

Supplemental Digital Content 2

Cell number correction of WST-1 reduction scores

We stained adherent cells per well by using Diff-Quick Staining Set, a staining set that yields results comparable to the Pappenheim technique (i.e. the cytoplasm of monocytes / macrophages is stained sky blue and nuclei are stained violet). The staining procedure was as

follows: After performing the WST-1 assay, the adherent macrophage layer was washed three times with 1 ml of warm (25°C) 0.01M PBS. Next, the adherent macrophage layer was overlaid with “Diff-Quick-Fix” solution for 30 seconds. After removing the “Diff-Quick-Fix” solution, the macrophage layer was overlaid with “Diff-Quick II” solution. After 30 seconds, the Diff-Quick II” solution was removed and the macrophage layer was overlaid with “Diff-Quick I” solution, again for 30 seconds. Then, “Diff-Quick I” was removed and the cell layer was washed three times with 1 ml of distilled water to remove excess stain. Stained cells were 40 times magnified and digitally photographed using a microscope with integrated camera (Nikon Eclipse TS1000, Nikon Industries, Egg, Switzerland). Stained cells of all participants were then counted from digital photographs by using Fiji ImageJ open source software (Schindelin et al., 2012).

	WST-1	corr WST-1	age	BMI	H-SBP	H-DBP	S-SBP	S-DBP	S-MAP	LDL / HDL	Creatinine	Sodium	Calcium	Potassium	HbA1c
WST-1	1.00	.86***	-.07	-.18	.52**	.30*	.38**	.36**	.38**	.12	.13	.13	.14	.01	.17
corr WST-1		1.00	-.03	.07	.39**	.28*	.35**	.34**	.35**	.10	.09	-.05	.12	.01	.13
age			1.00	-.01	-.08	.32	.08	.17*	.14	-.07	.00	-.10	-.01	.03	.28*
BMI				1.00	.51**	.30*	.48**	.47**	.49**	.35**	.16	-.32	-.16	.10	.04
H-SBP					1.00	.59**	.75**	.65**	.71**	.36**	.25	-.03	-.01	-.08	.12
H-DBP						1.00	.71**	.77**	.77**	.34**	.30	-.15	.25	-.10	.08
S-SBP							1.00	.88**	.96**	.34**	.23	-.13	-.20	-.27	.02
S-DBP								1.00	.98**	.33*	.25	-.21	.12	-.13	.10
S-MAP									1.00	.34**	.25	-.19	.02	-.19	.07
LDL/HDL										1.00	.02	.10	-.13	-.15	.21
Creatinine											1.00	.42	.00	.00	.02
Sodium												1.00	.00	-.30	-.07
Calcium													1.00	.07	.18
Potassium														1.00	.07
HbA1c															1.00

* p < .05, ** *p* < .01; *** *p* < .001

Notes: For H-SBP, H-DBP, LDL/HDL , and HbA1c n = 58; for creatinine, sodium, calcium, and potassium n = 27; WST-1 = WST-1 reduction score; corr WST-1 = WST-1 reduction score with correction for macrophage number ; BMI = body mass index; H-SBP = systolic blood pressure from home measurements; H-DBP = diastolic blood pressure from home measurements; S-SBP = systolic blood pressure from study measurements; S-DBP = diastolic blood pressure from study measurements; S-MAP = mean arterial blood pressure from study measurements.

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A4: HIGHER MACROPHAGE SUPEROXIDE ANION PRODUCTION IN CORONARY ARTERY DISEASE (CAD) PATIENTS WITH TYPE D PERSONALITY

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Abstract

Objective: Type D personality (Type D) is an independent psychosocial risk factor for poor cardiac prognosis and increased mortality in patients with cardiovascular disease (CVD), but the involved mechanisms are poorly understood. Macrophages play a pivotal role in atherosclerosis, the process underlying coronary artery disease (CAD). We investigated macrophage superoxide anion production in CAD patients with and without Type D.

Methods: We studied 20 male CAD patients with Type D (M: 66.7 ± 9.9 years) and 20 age-matched male CAD patients without Type D (M: 67.7 ± 8.5 years). Type D was measured using the DS14 questionnaire with the two subscales 'negative affectivity' and 'social inhibition'. We assessed macrophage superoxide anion production using the WST-1 assay. All analyses were controlled for potential confounders.

Results: CAD patients with Type D showed higher superoxide anion production compared to CAD patients without Type D ($F(1,38) = 15.57$, $p < .001$). Complementary analyses using the Type D subscales 'negative affectivity' and 'social inhibition', and their interaction as continuous measures, showed that both Type D subscales (negative affectivity: ($ß = .48$, $p = .002$, $R^2 = .227$); social inhibition: ($ß = .46$, $p = .003$, $R^2 = .208$) and their interaction ($ß = .36$, $p = .022$, $R^2 = .130$) were associated with higher WST-1 reduction scores. Results remained significant when controlling for classical CVD risk factors (i.e. body mass index, mean arterial blood pressure), atherosclerosis severity (i.e. intima media thickness, presence of carotid plaques), and psychological factors (i.e. depressive symptom severity, chronic stress).

Conclusions: Our results indicate higher macrophage superoxide anion production in CAD patients with Type D compared to those without Type D. This may suggest a mechanism contributing to increased morbidity and mortality in CAD patients with Type D.

1 INTRODUCION

Inflammatory processes play a pivotal role in the progression of coronary artery disease (CAD) and its underlying process atherosclerosis (Libby, 2002). Macrophages are tissue-resident phagocytic immune cells derived from circulating blood monocytes (Lucas & Greaves, 2001). A key initial event of coronary atherosclerosis is the entry of monocytes into the arterial intima, where they mature into macrophages. Intimal macrophages phagocyte oxidized lipoproteins and eventually differentiate into foam cells, a critical and prevalent component of atherosclerotic

plaques. Moreover, macrophages are important mediators of inflammation in atherosclerosis (Moore et al., 2013; Robbins et al., 2013).

Microbicidal activity, i.e. the killing of microbes, is a key innate immune effector function of classically activated macrophages also known as inflammatory M1 macrophages (Martinez et al., 2008; Mosser & Edwards, 2008). Macrophage microbicidal activity is largely mediated by increased secretion of microbe killing oxidizing agents termed reactive oxygen species (ROS; De Oliveira-Junior et al., 2011; Halliwell, 2006). ROS production in turn derives from activation of the multisubunit enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase located in the phagolysosome and plasma membrane of macrophages. Once activated, NADPH oxidase transfers electrons from NADPH in the cytosol to extracellular or intraphagolysosomal oxygen molecules. These oxygen molecules are then chemically reduced to highly reactive superoxide anions and other ROS subtypes (De Oliveira-Junior et al., 2011; Cathcart, 2004). In particular, superoxide anions are of major importance for the microbicidal activity of macrophages in host defense (Mosser & Edwards, 2008; Nathan & Shiloh, 2000).

Increasing evidence suggests that NADPH oxidase and the resulting production of superoxide anions are likely to play a critical role in the pathogenesis of atherosclerosis. For instance, NADPH oxidase-derived superoxide anions can induce low-density lipoprotein oxidation (Cathcart, 2004; Cai & Harrison, 2000; Griendling et al., 2000) an important cause of endothelial dysfunction as an initial step in atherosclerosis (Libby, 2002; Libby et al., 2011). Indeed, NADPH oxidase-deficient mice developed significantly smaller atherosclerotic lesions (Barry-Lane et al., 2001). Compared with healthy controls, CAD patients had greater NADPH oxidase activation and subsequent superoxide anion production in coronary arteries. This ROS increase was partly related to higher monocyte / macrophage infiltration (Guzik et al., 2006). Moreover, in CAD patients, superoxide anion production by stimulated neutrophils was enhanced with increasing arterial stiffness, a marker of adverse cardiovascular prognosis (Wykretowicz et al., 2005).

Different psychosocial factors significantly relate to the pathogenesis of CAD (Chida & Steptoe, 2009; Rozanski, 2014) and have been identified as risk factors for cardiac events both in healthy subjects and CAD patients (Chida & Steptoe, 2009; Lichtman et al., 2008). Type D ("distressed") personality (Type D) is defined as a tendency to experience negative emotions, and to inhibit their expression in a social context (Denollet et al., 1996). Evidence suggests that Type D is an independent predictor of cardiovascular morbidity and mortality in patients with CAD (Denollet et al., 2013; Grande et al., 2004; O'Dell et al., 2011). However, potential mechanisms underlying

poor cardiac prognosis with Type D are unclear and may include inflammatory processes (Denollet et al., 2003; Kupper & Denollet, 2007). Indeed, two studies with chronic heart failure (CHF) patients found Type D to independently predict increased circulating levels of tumor necrosis factor (TNF)-α and TNF-α soluble receptors (Conraads et al., 2006; Denollet et al., 2003). Of note, TNF-α is a pro-inflammatory cytokine involved in atherosclerosis development and progression (Libby, 2002) and also in the pathogenesis of CHF (Aukrust et al., 2005). Furthermore, in CHF patients Type D was associated with increased oxidative stress evidenced by assessing heat shock protein (HSP) 70 and xanthine oxidase (Kupper et al., 2009).

As yet, superoxide anion production in CAD patients with Type D has not been studied. Therefore, the aim of this study was to compare phagocytic NADPH oxidase-derived superoxide anion production by inflammatory M1 macrophages between CAD patients with and without Type D. To rule out potential confounding effects of age on superoxide anion production (Fulop et al., 2004, Shaw et al., 2013), we recruited CAD patients with and without Type D individually matched on age. Given the important role of both superoxide anion production and Type D in the pathogenesis and progression of CAD, we hypothesized that Type D patients would show higher superoxide anion production when compared with non-Type D patients.

2 MATERIALS AND METHODS

2.1 Patients with coronary artery disease

This study was part of a larger project assessing psychobiological mechanisms in patients with CAD. We contacted male patients who were diagnosed with CAD at the Cardiac Prevention and Rehabilitation Clinic, Bern University Hospital, Switzerland, at least 6 months previously. Patients interested to participate were screened by telephone interview using an extensive health questionnaire. Explicit exclusion criteria were: regular strenuous exercise, alcohol and illicit drug abuse, liver and renal diseases, chronic obstructive pulmonary disease, allergies and atopic diathesis, rheumatic diseases, HIV, cancer, psychiatric and neurological diseases, and current infectious diseases. Between December 2012 and June 2015, we enrolled a total of 101 male patients with a diagnosis of CAD who were also assessed for Type D (see below). For every patient classified as Type D, we recruited an age-matched CAD patient without Type D. Given well-known effects of age on various parameters of the immune system (e.g. Fulop et al., 2004; Shaw et al., 2013), we decided for an age-matched design allowing us to minimize potential confounding of macrophage superoxide anion production by age. In total, we identified 20 eligible CAD patients with Type D personality and recruited 20 eligible age-matched CAD

patients without Type D. For one 38-year-old CAD patient with Type D, we were unable to recruit an age-matched CAD patient without Type D; instead, we recruited an older control patient. Data of CAD patients were extracted from hospital charts and included the diagnosis of myocardial infarction (MI), left ventricular ejection fraction (LVEF), coronary artery bypass graft surgery (CABG), and the number of diseased coronary vessels (i.e., stenosis of at least 50%). Missing data regarding the number of diseased vessels from 4 patients were estimated using the expectation-maximization (EM) algorithm (Moon, 1996). The project was approved by the Ethics Committee of the Canton of Bern, Switzerland and the study protocol is in accordance with the Declaration of Helsinki. All study procedures were carried out with adequate understanding and written informed consent of all participants.

2.2 Procedure

Eligible CAD patients were asked to abstain from caffeine and alcohol consumption for 24 h before testing. They were briefed to consume a breakfast following standardized instructions prior to arrival at our lab at 0800 h. After patients had completed the Type D and other questionnaires, they provided blood samples obtained by short-term cannula insertion (see Kuebler et al., 2013) for the assessment of macrophage superoxide anion production at about 11:30h, (i.e., after a fasting period of 3.5h since arrival). In addition, participants underwent duplex ultrasonography for the assessment of intima media thickness (IMT) of the carotid common artery (CCA) and presence of carotid plaques as a measure of atherosclerotic disease severity. Moreover, blood pressure (BP) was assessed by means of sphygmomanometry (Omron M6; Omron Healthcare Europe B.V., Hoofdorp, Netherlands) 3h and 2.5h before and 10min after the blood samples were obtained. Due to technical problems, one CAD patient with Type D provided only two instead of three BP measurements.

2.3 Psychological Assessment

2.3.1 Type D Personality

Type D was assessed using the validated German version of the 14-item Type D Scale (DS14; Grande et al., 2004). The DS14 includes the two Type D subscales negative affectivity (NA, e.g. “I am often in a bad mood”) and social inhibition (SI, e.g. “I often feel inhibited in social interactions”) consisting of seven items each. Items are rated on a 5-point Likert scale (0=“false”, 1=“rather false”, 2=“neutral”, 3=“rather true”, 4=“true”). The total scores for each subscale range between 0 and 28. Type D is classified if both subscales are greater or equal to 10 (Denollet,

2005). Cronbach's alpha of the subscales was .88 for NA and .86 for SI (Denollet, 2005) and .89 for NA and .81 for SI in our sample. In addition, the interaction of continuous NA and SI Z scores was assessed as continuous single measure of Type D (Denollet et al., 2013).

2.3.2 Depressive Symptom Severity

Depressive symptom severity was measured using the validated German version (Hautzinger et al., 2006) of the 21-item Beck Depression Inventory – Second Edition (BDI-II; Beck et al., 1996). The BDI was developed for the assessment of depressive symptoms that correspond to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition criteria for major depressive disorders and measures a somatic and a cognitive-affective dimension of depression (Beck et al., 1996). The BDI-II assesses the frequency and / or severity of symptoms related to sadness, feelings of guilt, perceptions of self-worth, suicidal ideation, and changes in appetite and body weight, among other characteristics. Items are rated on a 4-point Likert scale ranging from 0 (symptom not present) to 3 (symptom very present) that add to a total BDI-II score ranging from 0 to 63. Higher scores mean higher depressive symptom severity. Cronbach's alpha of the BDI-II total score was between .84 and .91 for non-psychiatric populations (Hautzinger et al., 2006) and .86 in our sample.

2.3.3 Chronic Stress

To assess chronic stress we used the 12-item Chronic Stress Screening Scale (CSSS; Schulz et al., 2004). The CSSS includes questions about frequency of experiencing work overload (four items), worries (four items), lack of social recognition (two items), excessive demands at work (1 item) and social overload (1 item). Items have a 5-point rating format reflecting frequency (1="never" to 5="very often"). Possible scores range from 12 to 60 with higher scores indicating greater chronic stress. Cronbach's alpha of the CSSS total score was .87 (Schulz et al., 2004) and .94 in our sample. Missing CSSS scores of 3 patients with Type D were estimated using the EM algorithm (Moon, 1996).

2.4 Macrophage activation assessment

2.4.1 Reagents and Chemicals

We used the following reagents: Ficoll-Paque PLUS (Ficoll; no. 17-1440-02; GE Healthcare, Uppsala, Sweden), 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)2H-tetrazolium (WST-1; no. 150849-52-8; Dojindo Laboratories, Kumamoto, Japan), interferon-γ (IFN-γ; no.

PHC4031; Invitrogen, Basel, Switzerland), TNF-α (no. PHC3016; Invitrogen, Basel, Switzerland), Hank's balanced salt solution without phenol red (HBSS; no. 14025050; Invitrogen, Basel, Switzerland), fetal bovine serum (FBS; no. 10270-106; Invitrogen, Basel, Switzerland), lipopolysaccharide (LPS; no. L6529; Sigma-Aldrich, Buchs, Switzerland), phosphate-buffered saline (PBS; no. P5368; Sigma-Aldrich, Buchs, Switzerland), phorbol 12-myristate 13-acetate (PMA; no. P8139; Sigma-Aldrich, Buchs, Switzerland), and RPMI 1640 medium with glutamax (RPMI 1640; no. W9925E; Fisher Scientific, Wohlen, Switzerland).

2.4.2 WST-1 assay

We assessed superoxide anion production of *ex vivo* isolated human monocyte-derived M1 macrophages (HMDM) based on our recent assay validation procedure (Kuebler et al., 2013). In brief, 9ml of blood were collected in EDTA-coated tubes (Sarstedt, Numbrecht, Germany), immediately layered on top of 10ml Ficoll, and centrifuged for 20min at 300g and 20°C. After centrifugation, peripheral blood mononuclear cells (PBMCs) were removed from the interface, washed twice in warm RPMI-1640 medium, counted with a hematologic analyzer (KX-21N; Sysmex Digitana AG), and re-suspended to a final concentration of $3.0x10^6$/ml with RPMI-1640 media supplemented with 10% FBS. Then, PBMC suspension aliquots of 1 ml were transferred to 24-well cell culture plates (no. 4609; Semadeni; Ostermundigen, Switzerland). After incubation for 2 hours at 37°C and 5% CO_2, the supernatant was discarded and the plate surface was rinsed five times with 1ml of warm (25°C) 0.01M PBS to remove non-adherent PBMCs, while monocytes remained adherent to the bottom of the plate.

The adherent monocyte layer was then diluted with 1ml RPMI-1640 media supplemented with 10% FBS. Subsequently, we added IFN-γ, TNF-α, and LPS in a final concentration of 20ng/ml IFN-γ, 20ng/ml TNF-α, and 300ng/ml LPS to promote differentiation of monocytes into inflammatory macrophages. After incubation for 44h at 37°C and 5% CO_2, the supernatant was discarded and the adherent macrophage layer was washed twice with 1 ml of warm (25°C) 0.01M PBS to remove traces of culture media and non adherent cells.

Next, the resulting macrophage monolayer (obtained as described above) was overlaid with 1 ml HBSS. Subsequently, IFN-γ, TNF-α, LPS, WST-1, and PMA were added, resulting in a final concentration of 20ng/ml IFN-γ, 20ng/ml TNF-α, 300ng/ml LPS, 100M WST-1, and 50nM PMA. Following an incubation period of 4 hours at 37°C and 5% CO_2, the supernatant was removed and used to determine WST-1 reduction by reading the optical densities (ODs) with a spectrophotometer (Tecan infinite M1000, Tecan, Salzburg, Austria) at 450 nm against water as

blank. Higher ODs, as obtained in absorbance reading, are associated with higher amounts of WST-1 reduction and thus of superoxide anions generated by HMDM. In 2 CAD patients with Type D and 8 CAD patients without Type D the assays were not performed in duplicates due to low PBMC numbers.

2.5 Carotid ultrasound

Participants underwent carotid ultrasound at the Department of Neurology at the Bern University Hospital to assess CCA IMT (mm) and presence of carotid plaques (yes/no; Naqvi & Lee, 2014). A Toshiba Aplio 500 scanner (Toshiba Medical Systems, Nasu, Japan), equipped with a 12-MHz linear array transducer, was used by trained sonographers to image carotid arteries. High-resolution B-mode ultrasound images were collected from the far walls of the right and left CCA, and carotid bifurcation, whereby B-mode measurements were averaged over at least 1cm segment. Presence of carotid plaques was defined as focal region with carotid artery IMT greater than 1.5 mm that protrudes into the lumen distinct from the adjacent boundary.

In our analyses we controlled for the mean maximum IMT from far walls of right and left CCAs (mean max IMT) and for presence of carotid plaques.

2.6 Statistical analysis

Data were analyzed using SPSS (Version 20) statistical software package for Macintosh (IBM SPSS Statistics, NY, USA). All analyses were two-tailed, with level of significance at $p < .05$. Results are shown as mean ± SEM.

Prior to statistical analyses, linear data were tested for normal distribution and homogeneity of variance using Kolmogorov-Smirnov and Levene's tests in both groups. All data were normally distributed. For group comparisons WST-1 reduction scores had to be logarithmically transformed to verify homogeneity of variance. For reasons of clarity, we show original WST-1 reduction scores in all figures. We calculated mean arterial blood pressure (MAP) by the formula (2/3 mean diastolic BP)+(1/3 mean systolic BP). Body mass index (BMI) was calculated as the ratio of weight in kilograms to height in square meters.

We used analysis of variance (ANOVAs) to test for differences in the characteristics of the two groups and to test whether CAD patients with Type D showed higher WST-1 reduction scores as compared to CAD patients without Type D. Complementary analyses comprised multivariate linear regression analyses (enter method) with continuous measures of the Type D subscales and their interaction and tested whether WST-1 reduction scores (dependent variable) were associated

with NA, SI, or their interaction, respectively (independent variable). In all analyses with WST-1 reduction scores, we controlled in our age-matched participants for the classical CVD risk factors BMI and MAP as well as for the atherosclerosis severity parameters IMT and presence of carotid plaques. We additionally covaried for BDI and CSSS, because we previously found both these psychological variables to be related to macrophage superoxide anion production (Kuebler et al., 2013). We restricted the number of control variables of the main analyses to four in order to avoid overcontrolling given our sample size (Babyak, 2004). We selected these control variables a priori based on previous literature on their associations with immune activation or microbicidal superoxide anion production of HMDM, respectively (Dorshkind et al., 2009; Kuebler et al., 2013; Watanabe et al., 2006; Wirtz et al., 2008; Wirtz et al., 2004).

3 RESULTS

3.1 Group characteristics

Table 1 shows demographic, medical, and psychological characteristics of the 40 participants studied. The two study groups did not significantly differ in terms of age, BMI, MAP, IMT, or presence of carotid plaques. CAD patients with Type D had higher BDI and CSSS scores than those without Type D.

Table 1. Group characteristics of CAD patients with and without Type D personality

	CAD patients with Type D n = 20	CAD patients without Type D n = 20	*P*-value
Age (yrs)	66.7 ± 2.2	67.7 ± 2.4	.75
BMI (kg/m^2)	27.5 ± 0.6	27.8 ± 0.5	.80
Blood pressure			
SBP (mmHg)	136.1 ± 3.4	137.7 ± 4,14	.76
DBP (mmHg)	74.8 ± 2.0	79.0 ± 2.6	.21
MAP (mmHg)	95.2 ± 2.2	98.6 ± 3.0	.37
LVEF ≤ 40% (%)	4 (20)	6 (30)	.48
MI	8 (40)	12 (60)	.22
Number of diseased vessels			.20
1 (%)	5 (25)	10 (50)	
2 (%)	7 (35)	4 (20)	
3 (%)	8 (40)	6 (30)	
CABG (%)	5 (25)	6 (30)	.73
IMT (%)	0.85 ± 0.1	0.87 ± 0.2	.71
Plaques (%)	16 (80)	13 (65)	.30
Current smokers (%)	2 (10)	1 (5)	.56
Medication			
Antiplatelet drugs (%)	19 (95)	17 (85)	.30
Lipid-lowering drugs (%)	19 (95)	18 (90)	.56
Beta-blockers (%)	12 (60)	14 (70)	.52
ACE inhibitors (%)	6 (30)	8 (40)	.52

AT2 antagonists (%)	7 (35)	4 (20)	.30
Depressive symptom severity (BDI)	11.0 ± 1.8	5.2 ± 1.0	.009
Chronic stress (CSSS)	19.8 ± 1.7	9.4 ± 1.5	< .001

Notes: Values are means ± SEM; BDI = Beck Depression Inventory; BMI = body mass index; CABG = coronary artery bypass graft surgery; CAD = coronary artery disease; CSSS = Chronic Stress Screening Scale; DBP = diastolic blood pressure; IMT = intima-media thickness; LVEF = left ventricular ejection fraction; MAP = mean arterial blood pressure; MI = myocardial infarction; SBP = systolic blood pressure; SEM = standard error of mean; Type D = Type D personality

3.2 Type D groups and WST-1 reduction scores

Figure 1 shows that CAD patients with Type D had higher WST-1 reduction scores compared to CAD patients without Type D, either without ($F(1,38) = 15.57$, $p < .001$, $Eta^2 = 0.30$, $f = 0.65$) or with controlling for BMI and MAP ($F(3,36) = 14.45$, $p = .001$, $Eta_p^2 = 0.29$, $f = 0.64$). Additional controlling for IMT or presence of carotid plaques (*p's* = .001) did not significantly change results. Controlling for BDI, CSSS, or both BDI and CSSS, in addition to BMI and MAP did also not change results (*p's* ≤ .009), even if IMT or presence of carotid plaques were also controlled (*p's* ≤ .010).

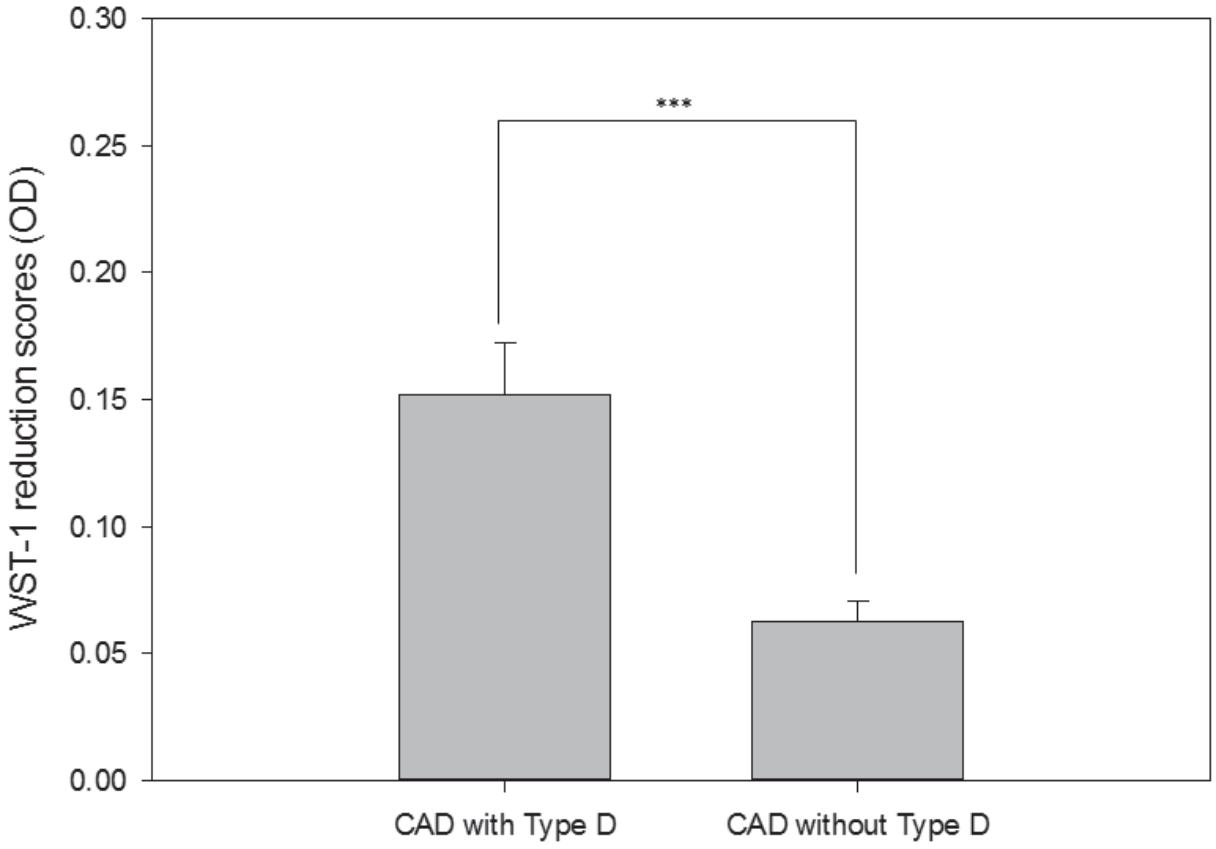

Figure 1. Group differences in WST-1 reduction scores

WST-1 reduction scores in coronary artery disease (CAD) patients with Type D personality (Type D) and CAD patients without Type D (mean ± SEM). CAD patients with Type D had higher WST-1 reduction scores than those without Type D ($p < .001$***).

3.3 Associations between WST-1 reduction scores and Type D subscales

Complementary analyses using the Type D subscales NA and SI, or their interaction as continuous measures showed that higher scores on both Type D subscales and their interaction were associated with greater WST-1 reduction scores without and with controlling for potential confounders.

3.3.1 Negative affectivity

In detail, higher NA was significantly associated with higher WST-1 reduction scores (*ß* = .48, $p = .002$, $R^2 = .227$; see Figure 2A). This effect remained significant after controlling for BMI and MAP (*ß* = .48, $p = .002$, $\Delta R^2 = .229$). Also, additional controlling for IMT or presence of carotid plaques ($p's \leq .003$) did not significantly change these results. Results remained significant after

controlling for BDI, CSSS, or both BDI and CSSS, in addition to BMI and MAP (*p's* ≤ .012), even if IMT or presence of carotid plaques were also controlled (*p's* ≤ .013).

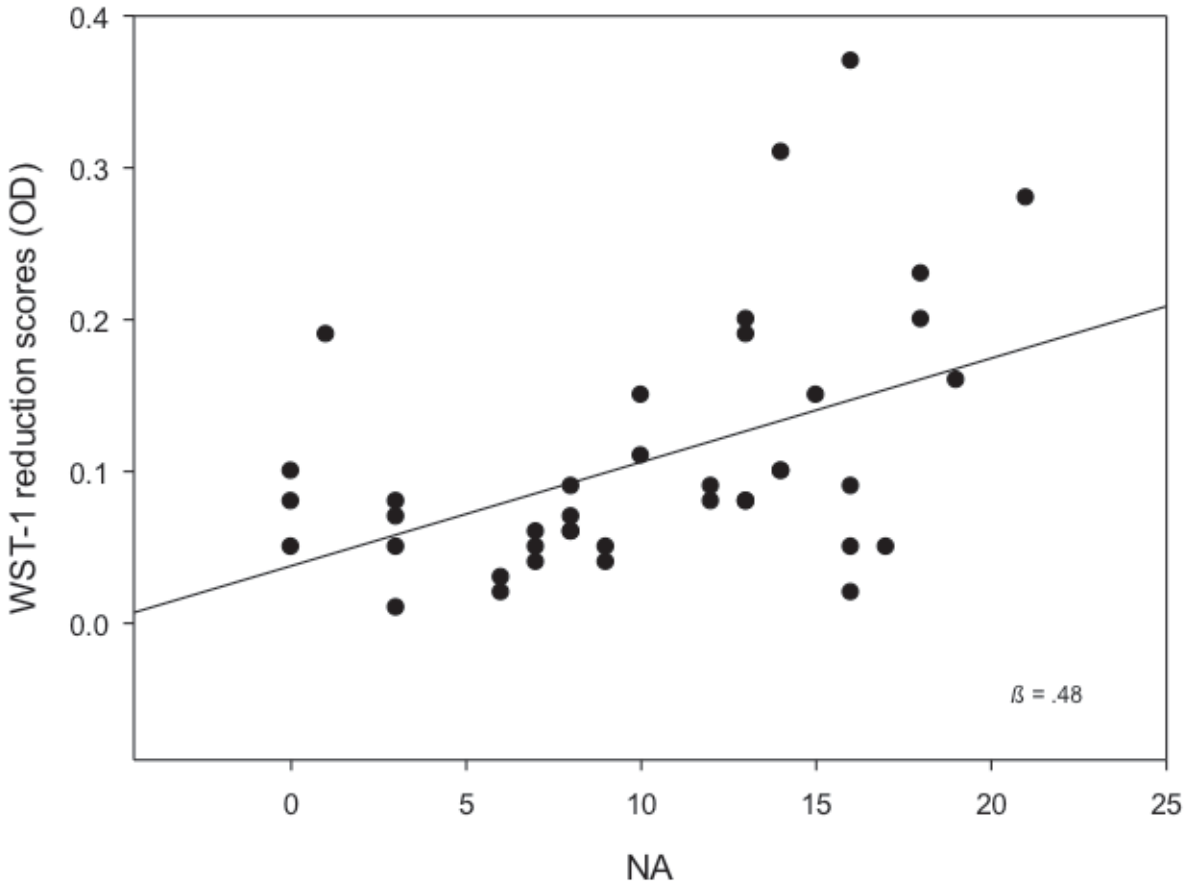

Figure 2A. WST-1 reduction scores in relation to negative affectivity (NA)

The scatter plot shows a linear positive relationship between negative affectivity (NA) and WST-1 reduction scores (p = .002).

3.3.2 Social inhibition

Similar results were obtained for SI: Higher SI significantly related to higher WST-1 reduction scores (*ß* = .46, p = .003, R^2 = .208; see Figure 2B). This effect was independent of BMI and MAP (*ß* = .45, p = .005, ΔR^2 = .197), even while also controlling for IMT or presence of carotid plaques (*p's* = .005). Again, these results held significance after controlling for BDI, CSSS, or both BDI and CSSS in addition to BMI and MAP (*p's* ≤ .023), also if adjusted for IMT or presence of carotid plaques (*p's* ≤ .025).

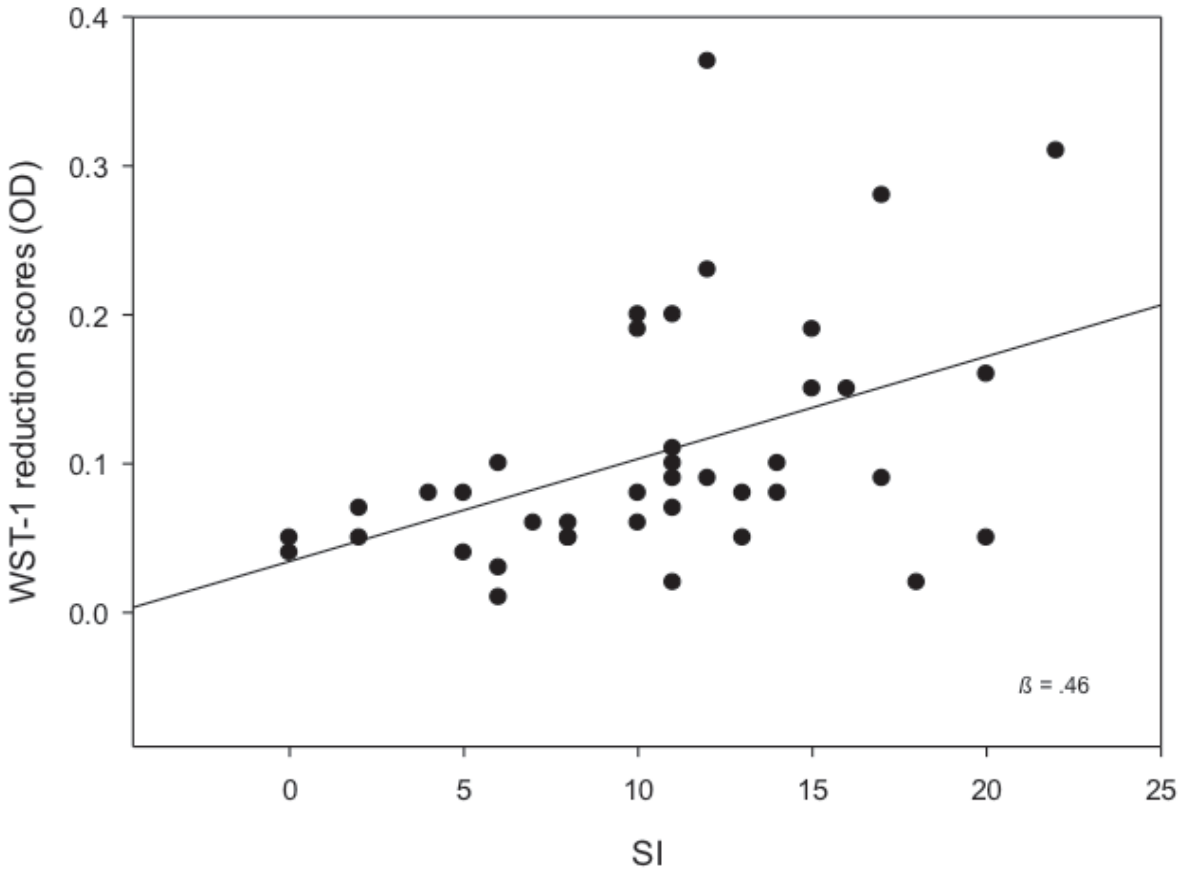

Figure 2B. WST-1 reduction scores in relation to social inhibition (SI)

The scatter plot shows a linear positive relationship between social inhibition (SI) and WST-1 reduction scores (p = .003).

3.3.3 Interaction negative affectivity and social inhibition

Regarding the interaction between NA and SI results showed that higher interaction scores significantly related to higher WST-1 reduction scores ($ß$ = .36, p = .022, R^2 = .130; see Figure 2C). This effect remained significant after controlling for BMI and MAP ($ß$ = .37, p = .032, ΔR^2 = .119). Additional controlling for IMT or presence of carotid plaques did not significantly change these results (p's ≤ .035). However, results became of borderline significance after controlling for BDI, CSSS, or both BDI and CSSS, in addition to BMI and MAP (p's ≤ .085), even if adjusted for IMT or presence of carotid plaques (p's ≤ .090).

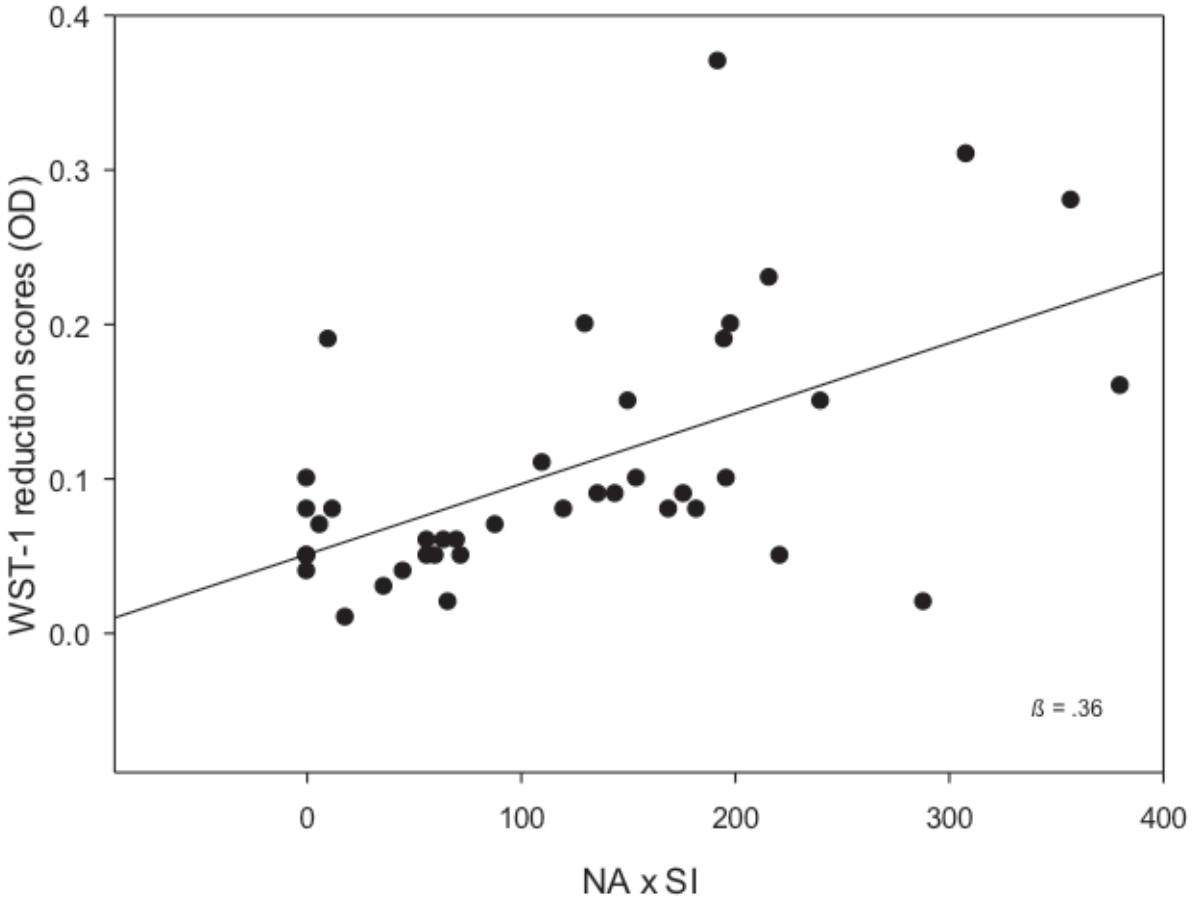

Figure 2B. WST-1 reduction scores in relation to interatction negative affectivity and social inhibition (NA x SI)

The scatter plot shows a linear positive relationship between the interaction of continuous negative affectivity and social inhibition (NA x SI) and WST-1 reduction scores (p = .022).

3.4 Asssociations between WST-1 reduction scores, chronic stress and depressive symptom severity

We further correlated WST-1 reduction scores with CSSS and BDI for the whole group in order to shed more light on the potential added value of Type D compared to depressive symptoms and chronic stress. Greater CSSS scores (WST-1 reduction score: ß = .30, p = .059, ΔR^2 = .090), but not BDI scores (WST-1 reduction score: ß = .23, p = .147, ΔR^2 = .055), were marginally associated with higher WST-1 reduction scores. Results remained similar after controlling for BMI, MAP, IMT, and presence of carotid plaques (CSSS: WST-1 reduction score: ß = .30, p = .085, ΔR^2 = .083; BDI: WST-1 reduction score: ß = .23, p = .186, ΔR^2 = .050).

4 DISCUSSION

We investigated for the first time phagocytic NADPH oxidase-derived superoxide anion production of inflammatory M1 macrophages in CAD patients with and without Type D. As hypothesized, we found that CAD patients with Type D had higher WST-1 reduction scores of PMA-activated *ex vivo* isolated monocyte-derived M1 macrophages and thus higher macrophage superoxide anion production than patients without Type D. In addition, both Type D subscales NA and SI and their interaction were associated with higher WST-1 reduction scores indicating higher superoxide anion production. Our results implicate that macrophages of CAD patients with Type D are characterized by an increased preparedness to release superoxide anions upon stimulation, i.e. in reaction to stimulating agents. The observed linear association with both Type D subscales suggests that this preparedness is higher with increasing NA and SI, respectively.

Our findings are in line with previous studies reporting associations between Type D and immune measures (Conraads et al., 2006; Denollet et al., 2003; Einvik et al., 2011). In healthy participants, the presence of Type D was associated with a higher prevalence of cardiovascular risk factors including BMI, serum triglyceride levels, and higher levels of the inflammatory acute phase protein C-reactive protein (CRP; Einvik et al., 2011). Furthermore, Denollet and colleagues (2003) and Conraads and colleagues (2006) found higher plasma levels of the pro-inflammatory cytokine TNF-α and increased TNF-α soluble receptors in CHF patients with Type D compared to non-Type D patients. Notably, higher TNF-α plasma levels relate to atherosclerosis development and progression (Libby et al., 2002; Kaptoge et al., 2014) and TNF-α soluble receptors may serve as a slow release reservoir for TNF-α. Moreover, in CHF patients, Type D was associated with increased oxidative stress in terms of higher serum levels of the oxidant marker xanthine oxidase together with lower serum levels of the antioxidant marker Hsp70 (Kupper et al., 2009). However, this study did not assess ROS production to measure oxidative stress. Our study extends these findings by pointing to phagocytic NADPH oxidase-derived superoxide anion production by inflammatory M1 macrophages in CAD patients with Type D.

Both inflammatory M1 macrophages and superoxide anions play a pivotal role in the pathogenesis of atherosclerosis (Moore et al., 2013; Robbins et al., 2013). Increased production of NADPH-derived superoxide anions by non-phagocytic cells has been identified as a characteristic feature of CAD promoting atherosclerosis (Cai & Harrison, 2000; Pennathur & Heinecke, 2007; Szocs et al., 2002), supposedly by increasing endothelial dysfunction (Cai & Harrison, 2000) and facilitating monocyte / macrophage infiltration into the intima (Guzik et

al., 2006). Notably, increased endothelial dysfunction may result as a "response-to-injury" induced by superoxide anion release in reaction to stimulation by oxidized LDL present in atherosclerotic lesions (Libby, 2002; Stocker & Keaney, 2004). Indeed, a very recent population-based study found Type D to be associated with endothelial dysfunction (van Dooren et al., 2016). In light of such reasoning, our findings may suggest a potential mechanism underlying the association between Type D and higher mortality in CAD patients.

We can only speculate about potential causes of the observed higher macrophage NADPH-derived superoxide anion production with Type D in CAD patients. Inflammatory processes have been discussed as a potential biological basis of poor cardiac prognosis with Type D in CAD (Einvik et al., 2011; Kupper & Denollet, 2007) and indeed, cardiac patients with Type D had higher basal inflammatory levels in terms of TNF-α (Conraads et al., 2006; Denollet et al., 2003). We speculate that inflammatory cytokines may prime macrophages to increased preparedness to superoxide anion release upon stimulation (Bedard & Krause, 2007; Gauss et al., 2007). To understand the process leading to higher basal cytokine levels in cardiac patients with Type D, it could be of interest to consider the two-stage model of vital exhaustion as proposed by Appels (1999). This model may also apply to Type D as a personality trait that likely predisposes to the experience of chronic stress. According to Appels (1999), overexertion or chronic stress leads to decreased immunocompetence (e.g. as a consequence of stress hormone secretion), thereby resulting in reactivation of systemic infections, and the release of cytokines.

Clinical implications of our findings may relate to therapeutic modulation of Type D. Although Type D is known to be a personality trait, it is conceivable that an individual's level of distress could be modified by eligible interventions (Bagherian-Sararoudi et al., 2012). Two studies have shown that expanded cardiac rehabilitation programs reduced Type D scores of both subscales (Karlsson et al., 2007; Sogaro et al., 2010) and improved the quality of life among patients with Type D (Karlsson et al., 2007). Thus, future research on the effects of psychological interventions aimed at improving Type D and related phagocytic NADPH oxidase-derived superoxide anion production is warranted.

Strengths of our study are the careful patient recruitment including age-matching of the Type D patient groups and the use of the well-validated WST-1 assay. Moreover, we controlled for a variety of potential CAD-related confounders such as carotid IMT, presence of carotid plaques, BMI, and MAP, as well as the hitherto known macrophage superoxide anion release related psychological factors depressive symptom severity and chronic stress. The study also has its

limitations. First, our study was cross-sectional and causality assumptions remain to be tested in prospective future investigations. Second, the present study focused on male CAD patients with and without Type D and may therefore not be generalizable to female patients with CAD or to patients with other diseases. Third, our experimental sample size requires confirmation in future studies with larger sample sizes. Fourth, the association of superoxide anion production as measured by the WST-1 assay with macrophage microbicidal activity in atherosclerotic lesions remains to be elucidated.

In sum, our data show an increased macrophage superoxide anion production in CAD patients with Type D compared to CAD patients without Type D. This suggests that macrophage superoxide anion production may play a mechanistic role in mediation of poor cardiac prognosis and higher morbidity and mortality in CAD patients with Type D.

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SUPPLEMENTAL MATERIAL

Supplemental Digital Content 1

Supplemental Table 1. Results of the models for the associations of the Type D scales (NA, SI), and their interaction (NAxSI) with WST-1 reduction scores adjusted for control variables

Type D subscale	Control variables	Standardized *ß-coefficient*	t-Value	*p*-Value	R^2 change
NA					
	BMI, MAP	.48	3.31	.002	.229
	BMI, MAP, IMT	.48	3.26	.003	.229
	BMI, MAP, plaques	.48	3.26	.002	.229
	BMI, MAP, CSSS	.60	2.71	.010	.157
	BMI, MAP, IMT, CSSS	.60	2.66	.011	.157
	BMI, MAP, plaques, CSSS	.60	2.68	.011	.157
	BMI, MAP, BDI	.50	2.90	.006	.181
	BMI, MAP, IMT, BDI	.50	2.86	.007	.181
	BMI, MAP, plaques, BDI	.50	2.85	.007	.180
	BMI, MAP, CSSS, BDI	.60	2.67	.012	.156
	BMI, MAP, IMT, CSSS, BDI	.60	2.63	.013	.156
	BMI, MAP, IMT, plaques, CSSS, BDI	.60	2.63	.013	.156
SI					
	BMI, MAP	.45	3.00	.005	.197
	BMI, MAP, IMT	.45	2.96	.005	.197
	BMI, MAP, plaques	.45	2.98	.005	.199
	BMI, MAP, CSSS	.40	2.37	.023	.125
	BMI, MAP, IMT, CSSS	.40	2.34	.025	.125
	BMI, MAP, plaques, CSSS	.40	2.35	.025	.126

	BMI, MAP, BDI	.42	2.73	.010	.165
	BMI, MAP, IMT, BDI	.42	2.70	.011	.165
	BMI, MAP, plaques, BDI	.42	2.71	.010	.166
	BMI, MAP, CSSS, BDI	.41	2.39	.023	.129
	BMI, MAP, IMT, CSSS, BDI	.41	2.36	.025	.129
	BMI, MAP, IMT, plaques, CSSS, BDI	.41	2.38	.023	.132
NA x SI					
	BMI, MAP	.37	2.23	.032	.119
	BMI, MAP, IMT	.37	2.20	.035	.119
	BMI, MAP, plaques	.40	2.30	.027	.129
	BMI, MAP, CSSS	.31	1.81	.079	.077
	BMI, MAP, IMT, CSSS	.31	1.78	.084	.077
	BMI, MAP, plaques, CSSS	.34	1.89	.068	.085
	BMI, MAP, BDI	.33	1.85	.073	.083
	BMI, MAP, IMT, BDI	.33	1.83	.076	.084
	BMI, MAP, plaques, BDI	.36	1.95	.060	.094
	BMI, MAP, CSSS, BDI	.32	1.76	.085	.076
	BMI, MAP, IMT, CSSS, BDI	.32	1.75	.090	.076
	BMI, MAP, IMT, plaques, CSSS, BDI	.34	1.84	.075	.084

Notes: BDI = Beck Depression Inventory; BMI = body mass index; CSSS = Chronic Stress Screening Scale; IMT = intima-media thickness; MAP = mean arterial blood pressure; NA = negative affectivity; NA x SI = interaction of continuous negative affectivity and social inhibition; SI = social inhibition