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"Myeloperoxidase is associated with cognitive function, brain morphology and subjective well-being in a cohort of elderly marathon runners"

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„The Language of Science is Broken English“

Unknown Origin

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At the beginning of scientific studies, I felt for learning how to find the right answers. Now, at the time of graduation, I know that it is even more important to identify the right questions. Thus, I want to thank a couple of people for enabling me to investigate into lots of interesting questions:

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ABSTRACT

Introduction: Myeloperoxidase (MPO) is a member of the mammalian heme peroxidase superfamily and is mainly expressed in cells of the myeloid lineage. The protein plays a crucial role in oxidative burst of activated neutrophil granulocytes and consequently in degradation of potential pathogens by production of reactive oxygen species. Again, myeloperoxidase leaked or spilled to the intercellular space affects host tissue as well. However, this can cause serious damage to key structures especially within the central nervous system. A G>A substitution at position -463 within the promoter region of the MPO gene results in a 25-fold decreased expression in cis by disruption of a binding site for transcription factor SP1. At the one hand, this may decrease oxidative stress, whereas at the other hand it can also lead to worse developed host protection systems against oxidative stress because of lower baseline MPO levels.

Methods: MPO-463G>A genotyping was done by realtime-PCR and allelic discrimination using TaqMan® fluorescence labeled probes, results were validated by restriction fragment length polymorphism analysis. Assessment of serum MPO was conducted by means of enzyme linked immunosorbent assays (ELISA).

Results and Discussion: Baseline MPO levels did not differ between athletes and controls, whereas high serum MPO levels seem to affect rather athletes than participants of the control group regarding executional cognitive functions. Moreover, we found a statistically significant correlation between MPO and signs of cerebral atrophy (CSF-space accentuation). Furthermore, MPO levels and genotype are associated with subjective well-being and Geriatric Depression Scale scores. Regarding the latter, high serum MPO is a higher risk in participants featuring the MPO[-463A] allele. This conforms to previous findings, which indicate that low baseline MPO levels can lead to reduced defense mechanisms against oxidative stress.

KURZFASSUNG

Einleitung: Myeloperoxidase (MPO), eine mammalische Häm-Peroxidase, wird hauptsächlich in Zellen der myeloiden Linie exprimiert. Das Enzym spielt eine maßgebliche Rolle im Rahmen der Oxidative Burst Reaktion aktivierter neutrophiler Granulozyten und damit im Abbau potentieller Pathogene. Dabei wird vordergründig H_2O_2 zu reaktiven Sauerstoffradikalen umgesetzt. Werden MPO oder dessen Endprodukte in den extrazellulären Raum entlassen, kann dies unter Umständen auch zu oxidativer Zerstörung des eigenen Gewebes führen. Dadurch können zentrale Strukturen innerhalb des zentralen Nervensystems nachhaltig geschädigt werden. Eine G>A Substitution an Position -463 innerhalb der Promotorregion des MPO-Gens führt zu einer fünfundzwanzigfachen Verminderung der Expression des Proteins, da eine starke Bindungsstelle für den Transkriptionsfaktor SP1 aufgelöst wird. Dadurch wird einerseits oxidativer Stress vermindert, dies wirkt sich damit aber auch auf den Aufbau von Schutzmechanismen vor oxidativem Stress aus.

Methoden: MPO-463G>A Genotypen wurden durch Sonden-PCR mit Endpunktanalyse (TaqMan®-Methode) ermittelt und durch Restriction Fragment Length Polymorphism Analyse bestätigt. Der Serum MPO-Spiegel wurde mittels Enzyme Linked Immunosorbent Assays (ELISA) gemessen.

Resultate und Diskussion: Der Basline-MPO Spiegel in AthletInnen unterschied sich nicht von dem in Kontrollpersonen, wobei sich höhere MPO-Konzentrationen stärker auf das Abschneiden von AthletInnen als von KontrollparticipantInnen in Kognitiven Tests auszuwirken scheint. Außerdem konnte ein Zusammenhang zwischen MPO und Zeichen zerebraler Atrophie (Liquorraumakzentuierung) gefunden werden. Weiters hängen Bewertungen des subjektiven Wohlbefindens und Scores der Geriatric Depression Scale mit MPO serum Konzentrationen und Genotypen ab. Bezüglich des Letzteren wurde festgestellt, dass hohe MPO-Spiegel nur in Trägern des MPO[-463A] Allels ein Risiko darstellen. Dies bestätigt die Hypothese, dass niedere MPO-Spiegel bei besagtem Allel auch geringere Abwehrmechanismen vor oxidativem Stress bedingen.

TABLE OF CONTENTS

1. INTRODUCTION

1.1. MYELOPEROXIDASE – HISTORY OF AN ENZYME

In the meantime, the discovery of myeloperoxidase dates back as many as 70 years[1] cited by [2], when Kjell Agner from the Serafimer Hospital in Stockholm, Sweden[3], identified an enzyme with peroxidase activity in leukocytes present in tubercular empyema and named it *Verdoperoxidase*. Further investigations on this enzyme took place hesitantly. However, this was due to difficulties in myeloperoxidase isolation resulting in only small amounts of the enzyme, hardly sufficient for extensive analyses[4] cited by [5]. As recently as the induction of a leukemic tumor called chloroma as an artificial source of leukocytes was possible in rats, production of sufficient amounts of the scarce protein was no longer a problem, since chloroma cells of young rats show a three-fold increased (6 %) concentration of myeloperoxidase, which moreover causes the green color of the solid tumor [5].

1.2. GENETIC BACKGROUND OF *MPO*

Myeloperoxidase (EC 1.11.1.7) is a member of the mammalian heme peroxidase superfamily and is therefore a homologue to lactoperoxidase and eosinophil peroxidase[6,7] cited by [2]. The coding regions of the three peroxidases are arranged next to each other at the q-arm of chromosome 17, suggesting a common origin via gene duplication[8] cited by [2] (see Fig. 1).

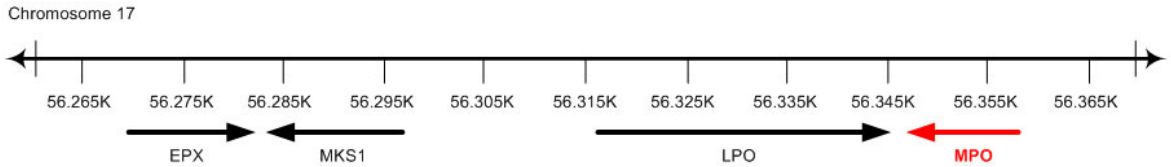

Figure 1: The homologue enzymes eosinophil peroxidase (*EPX*), lactoperoxidase (*LPO*) and myeloperoxidase (*MPO*) are coded on the q-arm of chromosome 17. There is evidence, that this three heme peroxidases derived from a common origin via gene duplication[8]. The protein of the coding region "Meckel Syndrome, type 1" (*MKS1*) is required for formation of the primary cilium in ciliated epithelial cells. Figure based on: [9], retrieved (15.03.2011) from http://www.ncbi.nlm.nih.gov/gene/4353#

Moreover, the promoter sequence of *MPO* contains a 5'-upstream single nucleotide polymorphism (NG_009629.1:g.4535G>A; NT_010783.15:g.21632914C>T; rs2333227; *MPO*-463G>A)[10]. Due to its critical position within a possible binding site of transcription factor SP1 and simultaneously within the first half-site of a retionoic acid receptor element (RARE)[11], this mutation is of special functional interest. In detail, position -505-200 represent a so-called *Alu*-element [12,13 cited by 11].

However, these elements make up about 5% of the human genome. These retrotransposons, genetically derived from *7SL*, have been spread over the entire genome via transcription by RNA polymerase III followed by reverse transcription and subsequent reintegration into the genome at many different sites, thus leading to development of seven distinct families of *Alu*-elements.[14,15 cited by 11]

Additionally, *Alu*-elements show regulative features regarding their genetic environment[16-21 cited by 11]. Thus, especially subclasses III and IV contain a hormone response element HRE[21 cited by 11], composed of hexamer motifs according to the consensus sequence AGGTCA. Hence the motifs can be recognized as a binding site for numerous nuclear receptors, e. g, vitamin D receptor VDR and steroid receptors as well as retinoic acid receptor RAR and thyroid hormone (T3) receptor TR[22-25 cited by 11]. Receptors of the RAR/TR family need retinoid X receptors RXR to form a heterodimer[26-30 cited by 11], thus binding direct repeats of two neighboring hexamer half-sites[31-33 cited by 11].

Preceding *MPO*, the said HRE consists of four half-sites reaching from position -463 to -437, again related to the consensus sequence AGGTCA. The first and the last two elements (orientated from 5' to 3' of the gene) form a motif called DR-2 and are separated by only two base pairs. Both of the middle hexamers make up DR-4, thus being separated by 4 base pairs. DR-2 represents a retinoic acid receptor element RARE, DR-4 is in turn typical for thyroid hormone response elements TRE. However, there is no evidence for in vivo importance of RAR or TR mediated regulation of *MPO*, but of transcription factor SP1, sharing a 10 bp consensus sequence within the very first DR-2 element.[11]

1.3. *MPO* AND TRANSCRIPTION FACTOR SP1

Not long ago, a sequence-specific regulation element, specificity protein 1 (SP1), has been found within the promoter of the tumor virus SV 40[34 cited by 35].
This transcription factor binds GC-rich sites, so-called GC-boxes by three C-terminal zinc finger-motifs, each consisting of a Cys_2His_2 sequence. Moreover, the protein consists of a buttonhead (Btd) box which plays a role in the transactivation activity of the transcription factor, glutamine-rich subdomains, which mediate the regulative activity, serine/threonine-rich target sites for post-translational modification as well as an N-terminal Sp box representing an endoproteolytic cleavage site.[36 cited by 35] In cells of the myeloid lineage, some twenty specific genes are known that feature GC-boxes, which thus can be regulated by transcription factor SP1 or its related factor SP3. [35] However, myeloperoxidase is one of it[11 cited by 35].

The previously mentioned *MPO*-463G>A single nucleotide polymorphism finally modifies hexamer 1 of the most 5' DR-2 motif within the promoter-Alu element at position 5. Interestingly, this alternation destroys a strong binding site for transcription factor SP1 leading to a 25-fold decrease in gene expression of *MPO* compared to the wild type promoter.[11]

1.4. *Expression of MPO – An Introduction*

The protein is mainly expressed in cells of the myeloid lineage, especially in precursors of neutrophil granulocytes. As shown in figure 2, transcription of MPO decreases with increased differentiation. Thus, myeloperoxidase is mainly synthesized in promyelocytes and promonocytes residing in the bone marrow[37 cited by 2].

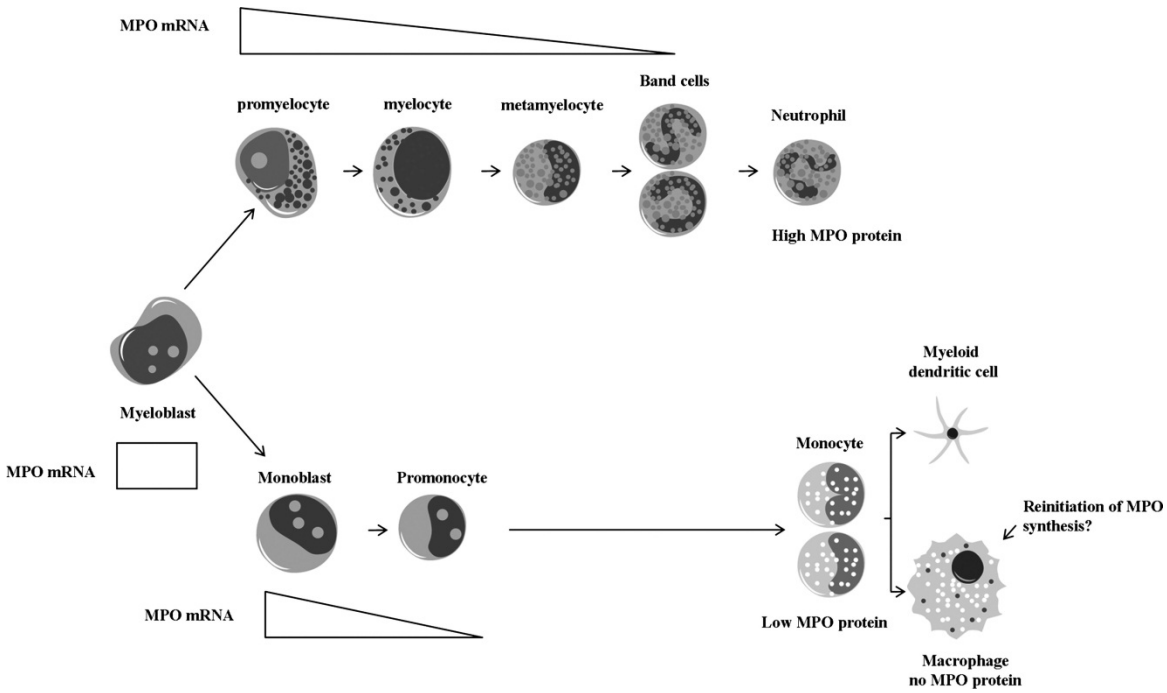

Figure 2: Expression of myeloperoxidase has been shown in a multitude of cell types related to the myeloid lineage, although in different concentrations. In detail, the amount of MPO mRNA decreases with the stage of cell differentiation. Subsequently, high levels of MPO mRNA can be found in myeloblasts, monoblasts and promyelocytes, whereas circulating cells contain little mRNA. Moreover, neutrophils contain high amounts of MPO protein compared to monocytes and tissue macrophages. Whether MPO synthesis is reinitiated in vivo in the latter has not been proven yet[37 cited by 2]. Figure obtained from: [2]

1.5. NEUTROPHIL GRANULOCYTES

To discuss the function of myeloperoxidase, it is inevitable to describe first its major source, the neutrophil granulocyte. Neutrophil granulocytes, hematopoetic cells of the myeloid lineage, are cells of the innate immune system produced from myeloblasts within the bone marrow by formation of different progenitor and intermediate cell types (see also figure 3) as a response to stimulation with granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF)[38,39 cited by 40].

Accordingly, neutrophil granulocytes and their precursors respectively can be found in different body compartments: the bone marrow, the circulation and in tissue in general. Cells residing in each pool thereby sustain a dynamic equilibrium of circulating cells, whereas young, so-called neutrophil band granulocytes migrate from the bone marrow to the circulation, where the total population of neutrophiles can be subdivided into a marginal and a circulating granulocyte pool[41 cited by 40]. Cells of the marginal pool reside at the endothelium and are of a significantly higher chronological age than circulating cells. However, this difference in cell age could be verified by measurement of granulocyte alkaline phosphatase activity in circulating blood before and after stimulation of cell migration from the marginal to the circulating pool. Granulocyte alkaline phosphatase activity is elevated in mature, polymorphnuclear granulocytes and can therefore be seen as an eligible marker for the mean chronological age of a neutrophil granulocyte population [42]. The third pool, the tissue neutrophiles, consists of cells that have penetrated the vessel wall[41 cited by 40].

1.6. NEUTROPHIL GRANULE COMPONENTS

Again, a morphological characteristic of neutrophils is the presence of different granules in the cytoplasm. These vesicles had already been described by

Metchnikov and Ehrlich as early as the turn of the 20th century [43,44 cited by 40]. In 1952, the physiological importance of the granules could be studied in patients' featuring abnormal granules as a result of the autosomal recessive Chediak-Higashi-Steinbrinck-syndrome[45 cited by 40]. Later, the role of neutrophil granules in phagocytosis was demonstrated by means of electron microscopy and phase contrast microscopy. In this context, the fusion of granules with membrane coated compartments originating from ingestion of extracellular particles resulting in a phagosome, could be shown[46,47 cited by 40].

The disparity between the types of granules results from the existence of different membrane and matrix proteins, each combination more or less specific for the respective kind of granule. In a nutshell, the granules can be traditionally subdivided into peroxidase positive (azurophil or primary granules) and peroxidase negative (secondary granules) vesicles[48,49 cited by 50], although in fact there is a vast multitude of different types regarding structure (content) and function (disposition for exocytosis or fusion with the phagosome) (Table 1) [50].

The heterogeneity of the granules arises from their time of production as proposed by the targeting-by-timing hypothesis, which reveals that expression of granule matrix and membrane proteins occurs staggered depending on the degree of maturation from promyelocyte to polymorphnucleic neutrophils. That means that granules assembling at the same time pack up more or less the same available combination of granule proteins[51 cited by 50]. However, consecutively expressed proteins like elastase underlie a sorting process what is yet not very well understood[52 cited by 50]. These different granules with their own specific content mediate the antimicrobial effect that neutrophils need to cope with their duties in cellular innate immunity [50].

Membrane Proteins			
Azurophil granules	**Specific granules**	**Gelatinase granules**	**Secretory vesicles**
N.a.	CD11b/CD18, CD66, CD67	CD11b/CD18, CD67	CD11b/CD18, CD67
N.a.	GP91phox/p22phox	GP91phox/p22phox	GP91phox/p22phox
N.a.		MMP26	MMP26
N.a.	TNFR[c], uPAR[c]	TNFR[c]	LIR1-4, -6, -7, -9[c]; CD35; CD16 C1q-R; IFN-αR1 and IFN-αR2[c]; IFN-γR1 and IFN-γR2[c]; TNFR1 and TNFR2[c]; IL-(1,4,6,10,13,17,18)R[c]; TGF-βR2[c]; CXCR-1[c]; CXCR-2[c]; CXCR-4[c]; CCR-1, -2, -3[c]; Ig(G,A,E)FcR[c]; TLR-1, -2, -4, -6, -8[c]; CD14, MyD88[c]; MD-2[c]; fMLPR, TREM1[c]
CD63, CD68 presenilin	SNAP-23, VAMP-2, Stomatin, PGLYRP[c]	SMA -23, VAMP-2, Nramp1	SNAP-23, VAMP-2, Nram 1, al aline phosphatase, DAF, CD10, CD13
Matrix Proteins			
Elastase, cathepsin G, proteinase 3	Collagenase, Gelatinase, uPA, cystatin C[c], cystatinF[c]	Gelatinase, arginase1	Plasma proteins
Defensis, BPI, MPO, lysozyme	hCAP18, NGAL, B12BP, lysozyme, lactoferrin, haptoglobin, pentraxin 3, prodefensin	Lysozyme	N.a.
Sialidase, 3-glucoronidase, azurocidin	α1-anti-trypsin[c], SLPO, orosomucoid, heparanase, β2-microglobulin, CRISP3	β2-microglobulin, CRISP3	N.a.

Table 1: Neutrophil granules and granule proteins[a,b] Modified table: [50].

"[a]Abbreviations: B12BP, vitamin B12 binding protein; CRISP, cysteine-rich secretory protein; DAF, decay-accelerating factor; Gp, granule protein; LIR, immunoglobulin-like receptor; n.a., not applicable; uPA, urokinase plasminogen activator.

[b]Localization of proteins in the matrix and the membrane of neutrophil granules and secretory vesicles. The list is not completely exhaustive but illustrates the major classes of proteins found in the various types of neutrophil granules. Yellow, adhesion molecules; blue, receptors; orange, antibacterial proteins; green, proteases; colorless, other functional classes of proteins.

[c]The localization is inferred from the gene expression profile according to the targeting-by-timing hypothesis but has not been confirmed at the protein level." [50]

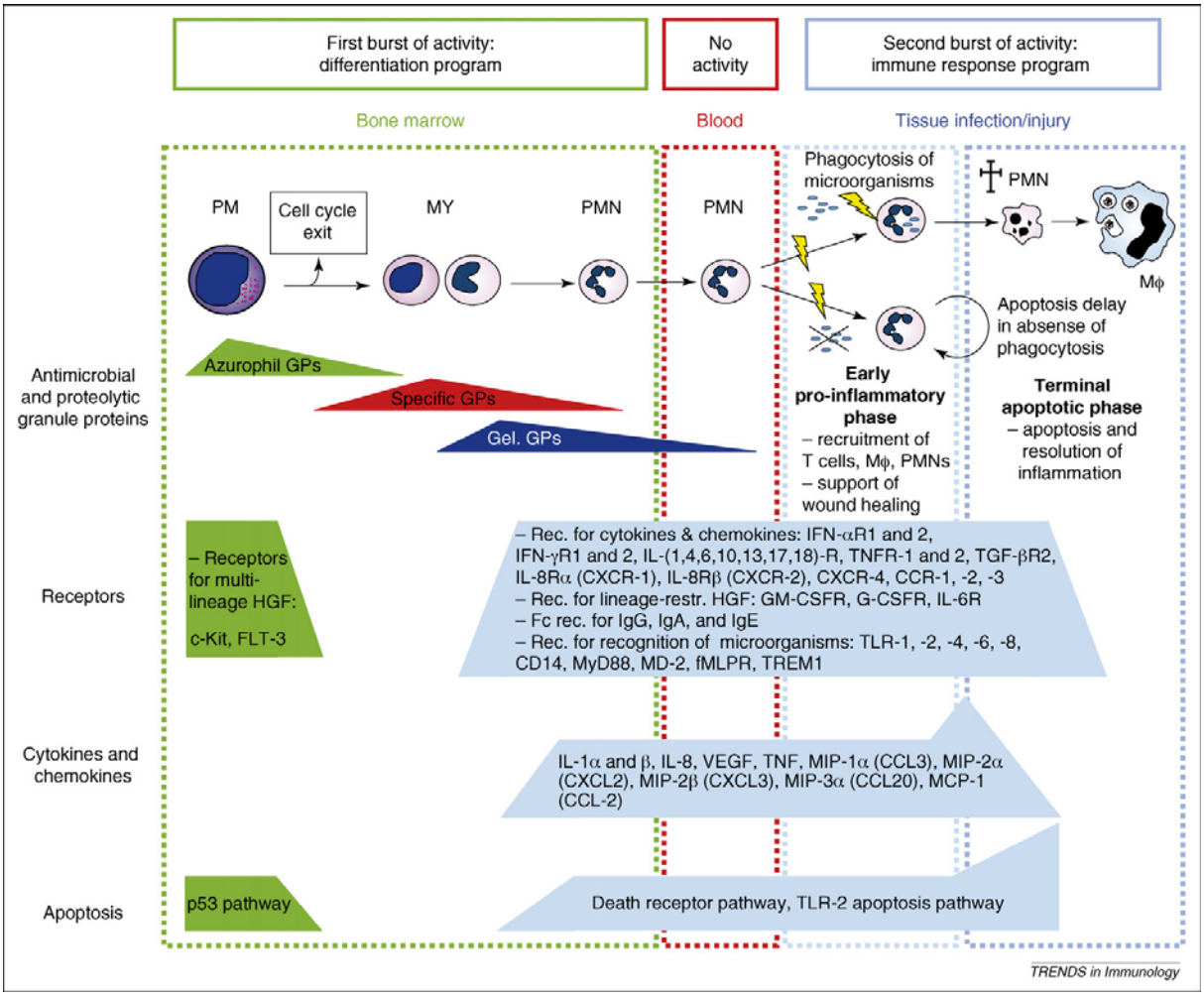

Figure 4: During their life cycle, neutrophil granulocytes meet two distinct bursts of gene expression. While the first occurs at the early myelopoetic phasis in myeloblasts (green bars), the latter happens mainly during diapedesis and phagocytosis (light blue bars). Moreover, sequential transcription of neutrophil granule proteins is expanded to myelocytes and metamyelocytes (red bars) as well as late metamyelocytes and early band cells (dark blue bars). Figure obtained from: [50]

"Abbreviations: FLT, FMS-like tyrosine kinase; G-CSF, granulocyte stimulating factor; GM-CSF, granulocyte-macrophage stimulating factor; HGF, hepatocytes growth factor; IL, interleukin; IFN, interferon; MCP, monocytes chemoattractant protein; MD, myeloid differentiation; Mf, macrophage; MIP, macrophageinflammatory protein; MY, myelocyte; PM, promyelocyte; PMN, polymorphonuclear granulocyte; TGF, transforming growth factor; TLR, Toll-like receptor; TNF, tumour necrosis factor; TREM, triggering receptor expressed on myeloid cells." [50]

Again, in literature on neutrophil transcription analysis, two bursts of transcriptive activity were described (see figure 3) [50]. The first burst was appointed during maturation within the bone marrow, including transcription of granule proteins, the p53 system and several receptors. The second burst occurs in the last stage of neutrophil life cycle: the exuded tissue neutrophil. During this, mainly cytokine/chemokine receptors, F_c receptors for recognition of antibodies' Fc (fragment crystallizable) domains, pattern recognition receptors (PRR), cytokines/chemokines and components for subsequent apoptotic pathways are produced.

1.7. Phagocytosis in Neutrophils

As already mentioned, a crucial physiological function of neutrophil granulocytes is their microbicidal effect, which mainly rests upon exocytosis of antibacterial peptides and proteases as well as phagocytosis with production of hypochlorous acid and reactive oxygen species[53,54 cited by 40]. The latter is done by peroxidases such as myeloperoxidase [55], which are stored in the cells' primary granules.

However, how does myeloperoxidase establish its microbicidal effects? To answer this question, we have to focus on the molecular processes occurring during a cellular innate immune response mediated by neutrophil granulocytes resulting in degranulation of primary granules. This process can either be induced by phagocytosis of antigens or by surfaces coated with complement molecules or antibodies. In the first case, the granule proteins and thus myeloperoxidase is released into the developing phagosome and therefore mostly stays inside the neutrophil, altough some enzyme can leak into the outside due to incomplete constriction of the early phagosome from the cell membrane. In the second case, however, the granule content is directly released to the outside[56 cited by 55].

In professional phagocytes such as neutrophil granulocytes, phagocytosis refers to a complex cycle from recognition of apoptotic cells [57] or a bacterial pattern [58-60], its degradation, and in case of the latter presentation on the cell surface by MHC-II complex to cells of the adapted immunity[61 cited by 58]. To simplify matters, phagocytosis of apoptotic cells will not be discussed below.

Thus, the process of microbe ingestion starts either with detection of a pathogen-associated molecular pattern (PAMP) by PRRs or recognition of an antigen, opsonized by complement or antibodies, by the respective complement receptor or Fc receptor[62]. Whereas both of the latter as well as some PRRs can trigger invagination of cell membrane and thus phagocytosis themselves, there is no fair evidence on the contribution of toll-like receptors (TLR), a diverse family of PRRs against a variety of molecular patterns[60], to endocytosis. Stadlbauer, Leendertsee and Lee [63-65] concluded, that absence of different TLRs does not influence phagocytic activity whereas Bellochio and Hayashi [66,67] found an increase in phagocytic activity after stimulation of TLR.

1.7.1. A Side Note on Toll-Like Receptors

In this connection, I assume that a short excurse to the field of TLRs will be apologized. However, currently thirteen distinct TLRs are known in mammal cells[68], each specified on the recognition of a small subset of antigens. They can be grossly classified into two subtypes depending on their location either on the cell surface or intracellular. The first subtype (TLR 1, -3, -4, -5, -6) is spezialised in detection of bacterial lipoprotein and lipoteichoic acid (TLR1+TLR2, TLR6), lipoprotein S (TLR2) and flagellin (TLR 5). The intracellular subtype includes receptors (TLR3, -7, -8, -9) focused on recognition of[69 cited by 70] mainly viral dsDNA (TLR3), ssRNA (TLR7, TLR8 [71]) and unmethylated CpG-DNA (TLR9)[69 cited by 70]. In neutrophil granulocytes, TLR3 is not expressed[67 cited by 70].

After recognition of the respective antigen by its receptors, a cascade resulting in the internatlization of the particle is started, whereas the molecular processes depend on the respective receptors involved. Engulfment of particles opsonized by complement is accomplished by simply sinking into the cell, whereas Fcγ-receptors induce active inclusion by formation of pseudopods surrounding the IgG-opsonized particle[72 cited by 62]. Subsequently, a great many of signaling events is started. Phagocytosis of unopsonized particles seems to be dependend on lipid rafts[73-75 cited by 72].

In case of FcR-mediated phagocytosis (see fig. 5), however, the first step in cell signaling is phosphorylation by a Src-family member of the cytosolic domain of the FCR or of associated proteins containing an immunoreceptor tyrosine-based activating motif (ITAM)[76,77 cited by 72]. Further signal transduction via phosphorylation is done by another tyrosine kinase. For instance, in IgG-mediated phagocytosis, Syk will be recruited[78,79 cited by 72], resulting in activation of several adaptor proteins including LAT[80 cited by 72], SLP-76, BLNK[81 cited by 72], Crk1 [82 cited by 72] and Nck[83 cited by 72] and Fyb/SLAP ([84] at that time unpublished data mentioned by [72]), as well as generation lipid mediators such as phosphatidylinositol-4,5-bisphospate[85 cited by 72] and 3'-phosphoinositides by phosphatidylinositol-3-kinase (PI3K)[72]. The latter is involved in activation of phospholipase c (PLC), which via production of diacylglycerol (DAG) in turn activates proteinkinase C (PKC) isoforms involved in particle uptake at the evolving phagosome[86 cited by 72]. Again, there is evidence that phosphoinositides and anionic phospholipids respectively play a key role in sustaining electrostatic interactions with polycationic protein motifs[87]. In terms of cytoskeletal remodeling, Rho-GTPase family members[72] (including RacI and Cdc42[88]) induce focal assembly of F-actin, whereas Gelsolin triggers its severing in order to get short filaments[72]. The first is supported by contribution of ARF6[89,90 cited by 72]; ARF6 further acts as a co-factor in PIP_2 generation[89 cited by 72]. Actin remodeling is also catalyzed by dephosphorylated Cofilin enhancing actin filament turnover[91,92 cited by 72].

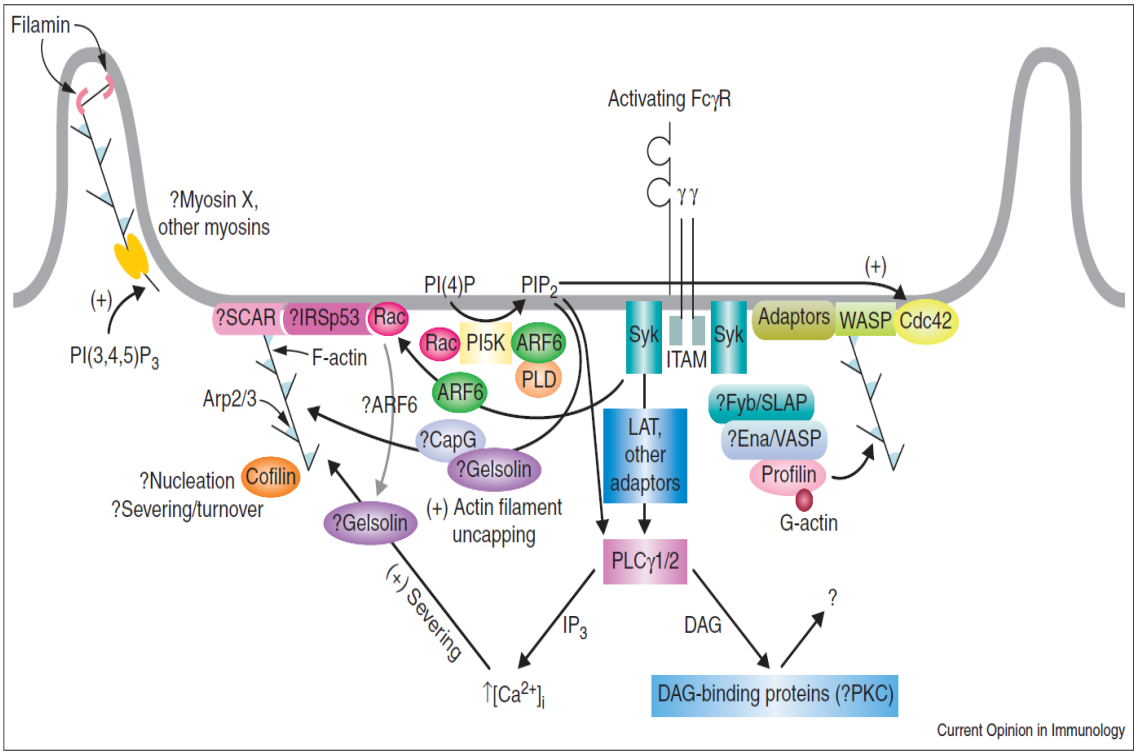

Figure 5: Depiction of signal transduction pathways in Fcγ-receptor mediated phagocytosis. Umpteen components are still hypothetical and unproven and thus indicated by "?". Figure obtained from: [72]

Indeed, it is a long way from a phagosomes' birth at the cell surface to its final destination as a matured phagolysosome. This transformation process contains changes in both, the chemical conditions within the phagosome lumen as well as the composition of protein[93]. The interchange of molecules between a maturing phagosome and other cell organelles has been described best by studying especially membrane-bound components. Hence, the investigations resulted in a decrease of cell surface markers coupled by an increase of protein typically for early and late endosomes as well as lysosomes[94,95 cited by 93] suggesting an onward fusion with the respective organelles[96 cited by 93], most likely by so-called "fusion/fission events"[93], better known as "kiss and run fusion"[93]. As suggested by Duclos et al.[97 cited by 93], these events could be regulated by Rab GTPases Rab5 and Rab7. Additionally, Rab7 is thought to bind its effector RILP[98 cited by 93], a protein attached to the microtubule-bound dynein-dynactin motor complex, resulting in anchoring at the microtubules system[99 cited by 93], which thus acts as a guide rail for maturing

phagosomes on their way into the inner cell[100 cited by 93]. During this, for instance proteins associated to lipid rafts[101 cited by 93] (flotillin-1, proton pump) as well as components of the NADPH oxidase[102,103 cited by 93], which is mainly involved in killing of ingested bacteria, are recruited to the phagosome.

Lipid components are estimated to play key roles in phagosome maturation. On that account, especially phosphatidylinositol 3-phosphates (PI(3)P) are held in high esteem. These lipids, most likely generated by the class III PI3K (phosphatidyinositol-3 kinase), have been identified to accumulate on the cytosolic leaflet of early endosomes[104 cited by 105] and interact with proteins containing FYVE and PX domains respectively[106,107 cited by 105] that contribute to phagosome maturation[105]. Moreover, PI3P is also involved in deactivation of Rab5[108 cited by 87]. Furthermore, changing the composition of membrane leaflets influences its electrostatic constitution by altered surface charge[87,109 cited by 87] thus causing changes in the recruitment of cationic molecules. This has been shown for phagosomal membrane layers during maturation[87].

The sense of this stepwise maturation is to progressively adjust the proteolytic and microbicidal conditions of the phagosomal lumen[93], including the previously mentioned fusion of myeloperoxidase positive granules with the phagosome. This brings us right back to the function of myeloperoxidase, which finally will be discussed below.

1.8. Chemical Properties and Reactions of Myeloperoxidase

The catalytic system of myeloperoxidase (figure 6A) consists of the enzyme itself, the oxidative species H_2O_2 and of course a reagent to be oxidized[2], for instance a bacterial component. However, hypochlorous acid (HOCl) leads instead of H_2O_2 to the same reaction[110 cited by 55]. The first step of the following redox-reaction includes the formation of compound I. In detail, generation of compound I occurs by oxidation

of the ferric heme center of MPO [MPO-Fe(III)] resulting in a oxy-ferryl group (MPO-FeIV=O). As a byproduct, H_2O_2 is reduced to H_2O.[2]

Compound I acts as the primary catalytic complex in further oxidations of halides and pseudohalides[111 cited by 2], alias halogenation cycle. During this, two electrons are transferred from the reduced oxy-ferryl group to halides resulting in the formation of hypohalous acids as well as the recycling of compound I to the ferric form of myeloperoxidase[6 cited by 2].

Of course, not only halides can act as reactants for myeloperoxidase compound I. Other reactants can be oxidized as well by the so-called peroxidase cycle[111 cited by 2]. This pathway includes the gain of a single electron from an oxidizable reactant generating compound II[6 cited by 2] containing an oxy-ferryl heme center without the π-cation radical of the porphyrin ring (MPO-FeIV-OH) resulting in a reduced activity[2]. Further oxidation of comparable reactant leads to reformation of ferric MPO [2] under abstraction of H_2O. Otherwise, regeneration of the native, active myeloperoxidase can be conducted[112-114 cited by 55] or at least accelerated[2] by exceeding superoxide radical $O_2^{-\cdot}$ (figure 6B).
This suggests a potentially regulative role of $O_2^{-\cdot}$ by maintaining the active form of MPO in an environment containing excess H_2O_2. In this case, oxidative damage at inflammatory sites would be increased by $O_2^{-\cdot}$ due to elevated production of HOCl by the regenerated enzyme in the presence of H_2O_2. This hypothesis can be supported by the anti-inflammatory effect of superoxide dismutase, an enzyme that converts $O_2^{-\cdot}$ to H_2O_2 and thus eliminates the superoxide radical.[112,114,115 cited by 55] However, the physiological importance of $O_2^{-\cdot}$ in activation of myeloperoxidase still leaves open questions, bearing the role of other reductants, as for instance ascorbic acid in mind, which show a considerably faster conversion of compound II to the active state [116,117 cited by 55] than the superoxide radical.

A third intermediate of myeloperoxidase, compound III (MPO-$Fe^{2+}O_2$), can be derived either from compound II by reaction with H_2O_2 or from native ferric state in presence of $O_2^{-\cdot}$ [2]. This unstable compound III, however, shows a half-decay time of

only a few minutes at room temperature, rather quickly decaying to the ferric state[118,119 cited by 55]. Nevertheless, there is evidence that even this unstable intermediate could act as a catalytically active kind of myeloperoxidase. That arises from the fact, that compound III is present in intact, stimulated neutrophils[120 cited by 55] and that it potentially reacts with electron donors[119,121-123 cited by 55] as well as acceptors[123 cited by 55].

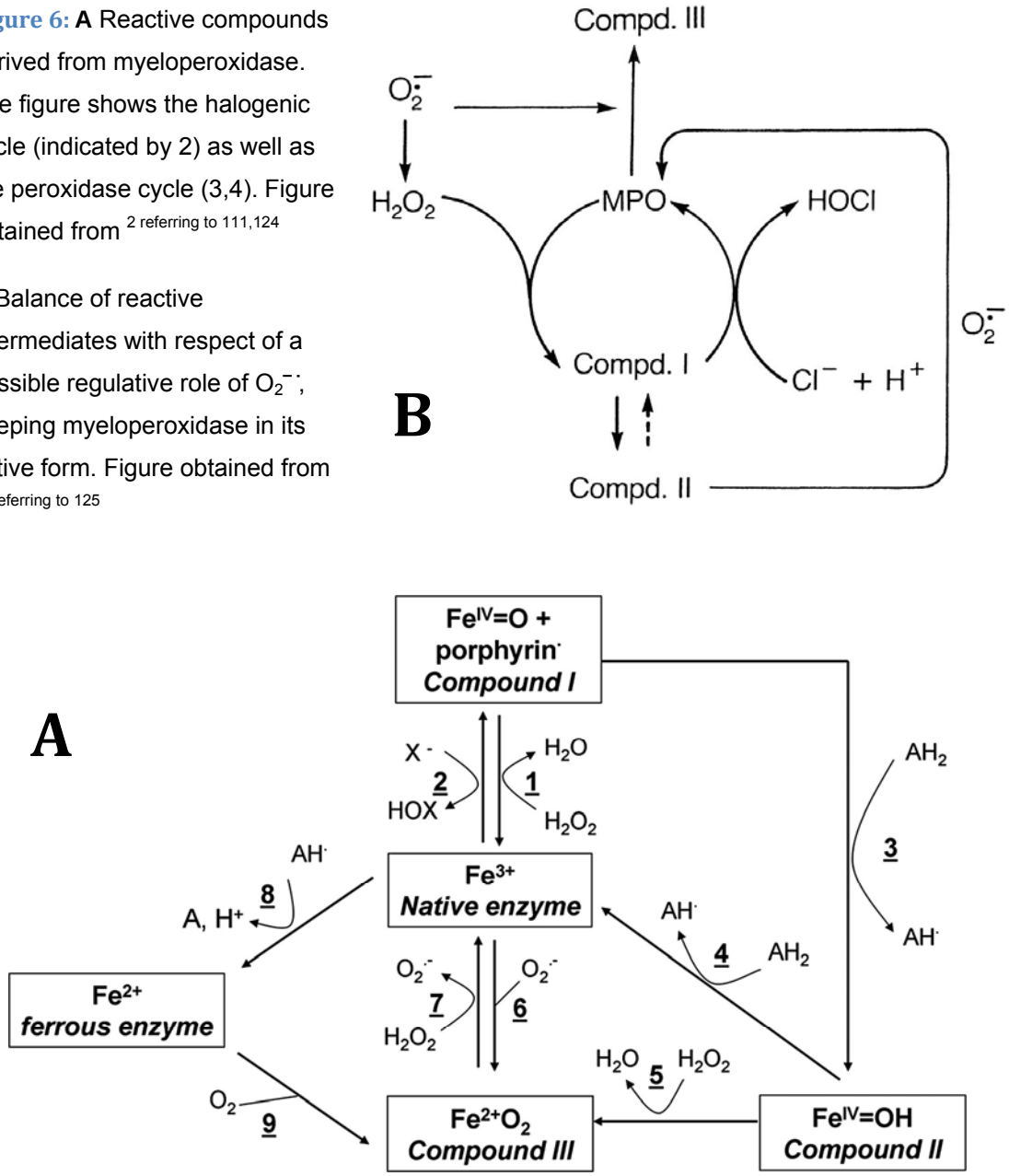

Figure 6: **A** Reactive compounds derived from myeloperoxidase. The figure shows the halogenic cycle (indicated by 2) as well as the peroxidase cycle (3,4). Figure obtained from [2 referring to 111,124]

B Balance of reactive intermediates with respect of a possible regulative role of $O_2^{-\bullet}$, keeping myeloperoxidase in its active form. Figure obtained from [55 referring to 125]

1.9. THE RESPIRATORY BURST OXIDASE – WHERE DOES ALL THAT H_2O_2 COME FROM?

As mentioned before, the function of myeloperoxidase is closely linked to the presence of hydrogen peroxide H_2O_2. Consequently, we need to ascertain possible sources of this major component. In his latest review on myeloperoxidase, Seymour Klebanoff[55] proposes several resources, some possibly contributing more to the physiological H_2O_2 concentrations available for myeloperoxidase than others.

1.9.1. PHAGOCYTIC SOURCES OF H_2O_2

However, the phagocyte NADPH oxidase complex is considered as an exceedingly important provider (figure 7), as we learn from patients suffering from chronic granulomatous disease CGD, which have to sustain recurring infections and formation of granulomas consisting of fused monocytes and macrophages, that are deficient in elimination of their ingested pathogens[126 cited by 127].

The protein complex is made up of four oxidase-specific components termed $p22^{phox}$, $p47^{phox}$, $p67^{phox}$ and $gp91^{phox}$ (whereas *phox* abbreviates phagocyte oxidase) as well as a Rac1/2 GTPase and exists in combination with regulative $p40^{phox}$ and Rap1A [128 cited by 127]. $gp91^{phox}$ is supposed to contain flavin and heme as a binding side for NADPH, which is required for further electron transportation[55]. However, together with $p22^{phox}$ this compound has been shown to form a membrane-bound heterodimer known as b-cytochrome[129 cited by 55].

Again, the b-cytochrome heterodimer holds adequate physical features (absorption peak at 558 nm) for oxidation of molecular O_2 and subsequent generation of $O_2^{-\cdot}$. Upon stimulation of neutrophils, b-cytochrome residing in membranes of specific granules and secretory vesicles is introduced into cell membrane or phagosomal membrane, hence fusing with the cytoplasmic components $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$. Thus activated NADPH oxidase forms $O_2^{-\cdot}$ by transferring an electron across the membrane to O_2 residing at the cell surface or the phagosome lumen respectively. [55]

Featuring a pK_a of 4.8, consequently at a pH of 4.8, $O_2^{-\cdot}$ occurs in equal parts with its protonated species $HO_2^{\cdot}$. At the same time, spontaneous dismutation via formation of O_2 and H_2O_2 by reaction of two molecules $O_2^{-\cdot}$ occurs, thus generating our desired hydrogen peroxide for the first time. At a higher pH, the velocity of H_2O_2 formation by spontaneous dismutation radically lags behind the rate achieved by superoxide dismutase (SOD)-catalysed reaction. However, there is no evidence for transportation of SOD into the phagosome, although the enzyme could be part of the ingested pathogen. [55]

1.9.2. EXTERNAL SOURCES OF H_2O_2

Mitochondrial metabolism producing small amounts of H_2O_2 could serve as another source of hydrogen peroxide for the myeloperoxidase system. However, whether the oxidant is released from its origin remains still unclear.

Additionally, a NAD(P)H oxidase present in vascular cells[130-135 cited by 55] is supposed to provide H_2O_2 for myeloperoxidase released to the blood stream thus dripping into the endothelium. There, the myeloperoxidase system is considered to play a role in vascular signaling via formation of nitric oxide.[136 cited by 55]

Moreover, non-phagocytic homologues of the phagocyte NADPH oxidase known as NOX (NADPH oxidase) or DUOX (dual oxidase) enzymes[137,138 cited by 55] could also somehow contribute reactive oxygen species (ROS) to the myeloperoxidase system[55].

Soluble oxidases as for example xanthine oxidase , amine oxidase and glucose oxidase have been shown to form H_2O_2, whereas the first is involved in ROS production causing reperfusion injury to ischemic tissue[139 cited by 55].

Another possible source of H_2O_2 could be represented by lactic acid bacteria[140 cited by 55], e. g. certain strains of pneumococci, streptococci and lactobacilli as well. [55]

However, the latter act as physiologic symbiotes at the vaginal mucosa. The immunological importance of lactobacilli as a microbial source of H_2O_2 is supported by the finding, that women harboring strains of lactobacilli that are not capable of forming H_2O_2 are significantly predisposed for bacterial vaginosis[141 cited by 55], as well as several other infections of the urogenital tract[142 cited by 55].

1.10. Microbicidal effect of Myeloperoxidase-Derived Oxidants

The microbicidal effect of myeloperoxidase is mainly due to generation of three specifiable groups of oxidants by its catalytic complexes, depending on its respective reactant Cl^-, NO_2^- or tyrosine[2] (figure 7).

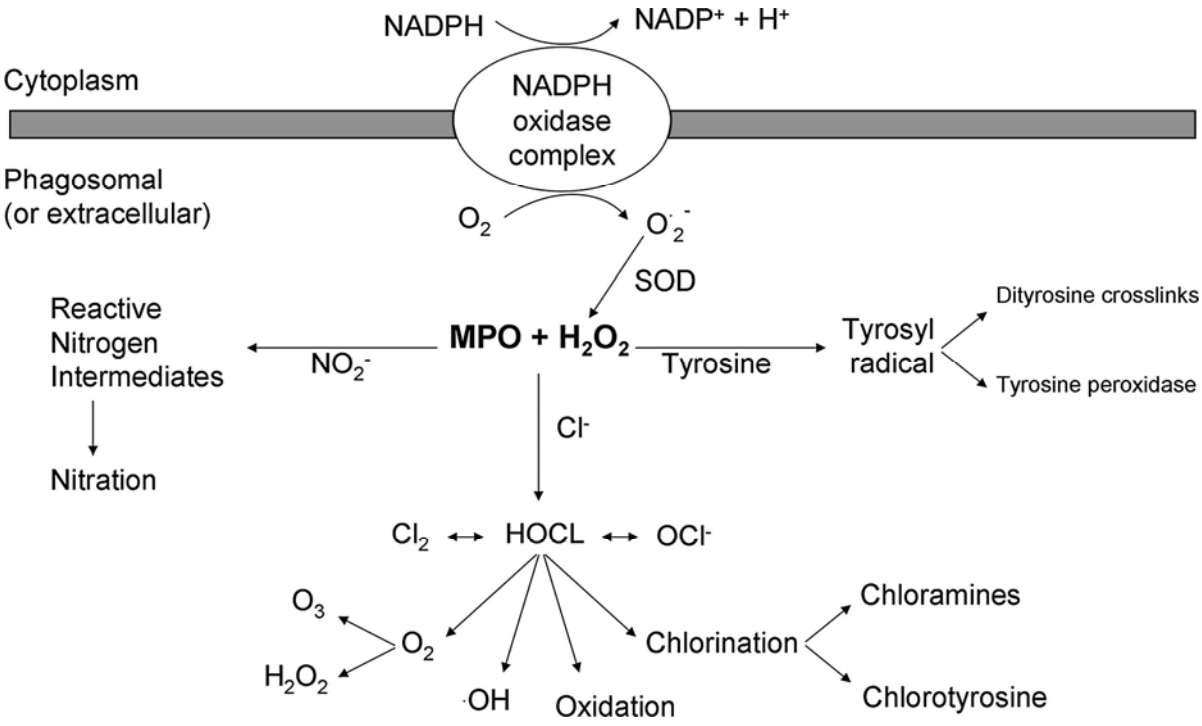

Figure 7: The NADPH-oxidase complexes residing at the cell membrane and the phagosomal membrane respectively (possibly together with superoxide dismutase SOD) keep supplies of H_2O_2 for the myeloperoxidase system coming. Moreover, formation of different microbicidal compounds by the myeloperoxidase + H_2O_2 system depends on the respective reactant Cl^-, NO_2^- or tyrosine. Figure obtained from [2]

Related to the first reactant Cl^-, production of the following highly reactive components is enabled, which will be further discussed: e. g. hypochlorous acid HOCl, chloramines, hydroxyl radicals $HO^{\cdot}$, ozone O_3 and singlet oxygen $^1O_2$ [55,111 cited by 2].

As a weak acid featuring a pK_a of 7.53, HOCl deprotonates incompletely, under standard conditions resulting in a composition of the acid and its anionic base, hypochlorite OCl^-. A decrease in pH, as it progressively occurs during phagosome maturation, thus leads to a shift in favor of the acidic species. [55]

When HOCl reacts with excess chloride, highly reactive[143cited by 55] molecular chlorine (Cl_2) will be generated[144-146 cited by 55]. Subsequently, this molecule is able to oxidize a plethora of functional groups present on microbes, including sulfhydryl and sulfur-ether groups, iron-sulfur centers, heme as well as unsaturated fatty acids[55]. Accordingly, multiple metabolic functions are affected: loss of membrane transport[147]

cited by 55, and adenylate energy reserves[148 cited by 55] as well as demolition of electron transport chain at the microbial membrane[149 cited by 55] and elimination of DNA synthesis, e.g. by detaching the chromosomal origin from the bacterial cell membrane[150 cited by 55]. Additionally, extension of antimicrobial activity in time and space is conducted via durable chloramines. These compounds are formed by reaction of HOCl with nitrogen-containing molecules and occasionally degrade to the respective aldehyde[151,152 cited by 55], maintaining their oxidizing properties. However, damage by HOCl leaking to the neutrophil cytoplasm can be diminished by high amounts of taurine[153,154 cited by 55] via reaction to taurine chloramine, a less reactive and hence toxic compound. This could be considered as a technique by which the proverbial snake bears its own poison, whereas including more recent findings one could tend to put this possible protective mechanism in question[2]. More precisely, taurin is classified as a possibly cytotoxic, long lived oxidant[155 cited by 2], selectively targeting creatinine kinase, glyceraldehyde-3-phosphate dehydrogenase and critical cysteins[156 cited by 2].

Small amounts of $HO^{\cdot}$ produced by the myeloperoxidase system[157 cited by 55] result from HOCl reacting with $O_2^{-\cdot}$ generating O_2 and Cl^- as by-products[55]. However, this compound is extremely reactive and thus features just a slight spatial radius. Forming $HCO_3^{\cdot}$ by reaction with CO_2, another antibiotic oxidant has been produced that finds its preferred environment within the phagosome.[158 cited by 55]

Formation of $^1O_2$ by the myeloperoxidase system according to $H_2O_2 + OCl^- \rightarrow {}^1O_2 + H_2O + Cl^-$ has be shown experimentally under physiological conditions[159 cited by 55], whereas the situation in vivo still needs further evaluation[55]. O_3 is further supposed to be produced by neutrophil respirative burst with consumption of $^1O_2$ [160 cited by 55]. Moreover, the microbocidal effects of H_2O_2 and O_3 reinforce each other[55].

Secondly, inflammation features an increase of NO_2 (>200 µM) within the affected environment, providing a rich variety of reactants for myeloperoxidase compound I [161-163 cited by 2]. Nitrite NO_2^- and myeloperoxidase + H_2O_2 react to the unstable compound nitrogen dioxide radical $NO_2^{\cdot}$, which promotes lipid peroxidation and nitration of proteins[163-169 cited by 2], as well as oxidation of the amino acid tyrosine, thus

producing nitrotyrosine, a marker of oxidative stress[170-172 cited by 2]. The latter is again attributed to inflammatory cellular dysfunction[163,165 cited by 2].

Tyrosine as a third direct reactant of myeloperoxidase and H_2O_2 can thus be converted into the tyrosyl radical in absence of Cl^-[55]. Subsequently, this compound can form either free or protein-dityrosine linkages [173,174 cited by 55]. Furthermore, tyrosine peroxide can be produced from tyrosyl radical reacting with superoxide[175 cited by 55].

1.11. Pathophysiological Importance of MPO

As reported by van der Veen et al.[2], the prevalence of hereditary MPO deficiency in Europe and the United States of America ranges between 1:1000 and 1:4000[176-180]. Indeed, these individuals show significantly higher incidences of infectious as well as chronic inflammatory diseases[181 cited by 2], although the reported patients were not "extremely more susceptible to infections" [181 cited by 2] as it is known for patients suffering from chronic granulomatous disease, expressing a dysfunctional NADPH oxidase thus lacking sufficient generation of H_2O_2 [182 cited by 2]. Moreover, the microbicidal activity of MPO deficient neutrophils exceeds that of neutrophil granulocytes treated with peroxidase inhibitors[183 cited by 2]. However, these findings indicate a possible compensation of a dysfunctional MPO-system in-vivo by MPO-indipendent mechanisms [2].

1.12. MPO and Cognitive Decline

"The term *cognitive decline* actually reflects a continuum of cognitive changes; some are considered to be within the spectrum of normal aging, whereas others exceed expected decline and are categorized as mild impairment."[184]
However, the line between normal and pathologic decline, also referred as mild cognitive impairment (MCI) or cognitive impairment without dementia, are blurred.

Usually, diagnosis of MCI occurs via testing and subsequent evaluation of cognitive domains such as language, orientation, memory and executive functions. [184] Moreover, cognitive decline may pose a risk for progression to MCI and dementia[184], whereas cognitive decline and dementia overlap or more exactly "may represent a continuum of disease"[185 cited by 186].
To the patients suffering from it, cognitive decline means a certain condition of elevated disability and thus decreases autonomy and quality of life. However, said condition shows multiple reasons ranging from biological to environmental, social and even behavioral factors and most of them remain still unknown. [184]

A potential contribution of oxidative stress factors to cognitive decline has been suggested by numerous publications. Thus, production of free radicals during oxidative processes may promote cognitive impairment by either increased atherosclerosis and thrombosis[187,188] or selective brain damage[189]. The latter could be due to a special sensitivity of brain tissue to oxidative stress that arises from its oxygen metabolic rate which is the highest throughout the body, its high concentration of unsaturated fatty acids which can be easily modified by free radicals and finally by its high level of iron, an element that correlates with injury caused by free radicals[190]. In contrast, the tissue features respectively low amounts of antioxidant agents and enzymes[190].

As mentioned in chapter 1.10, MPO moreover catalyzes the oxidation of halides and thiocyanate (SCN^-), a pseudohalide, to the respective hypohalous acids[191 cited by 190] resulting in "chlorinative stress"[190] to the surrounding tissue (see fig. 8). Interestingly, these reactions may also occur in the brain and are associated with several neurological diseases, as for example Parkinson's disease, Alzheimer's disease, Multiple Sclerosis and cerebral ischemia[190].

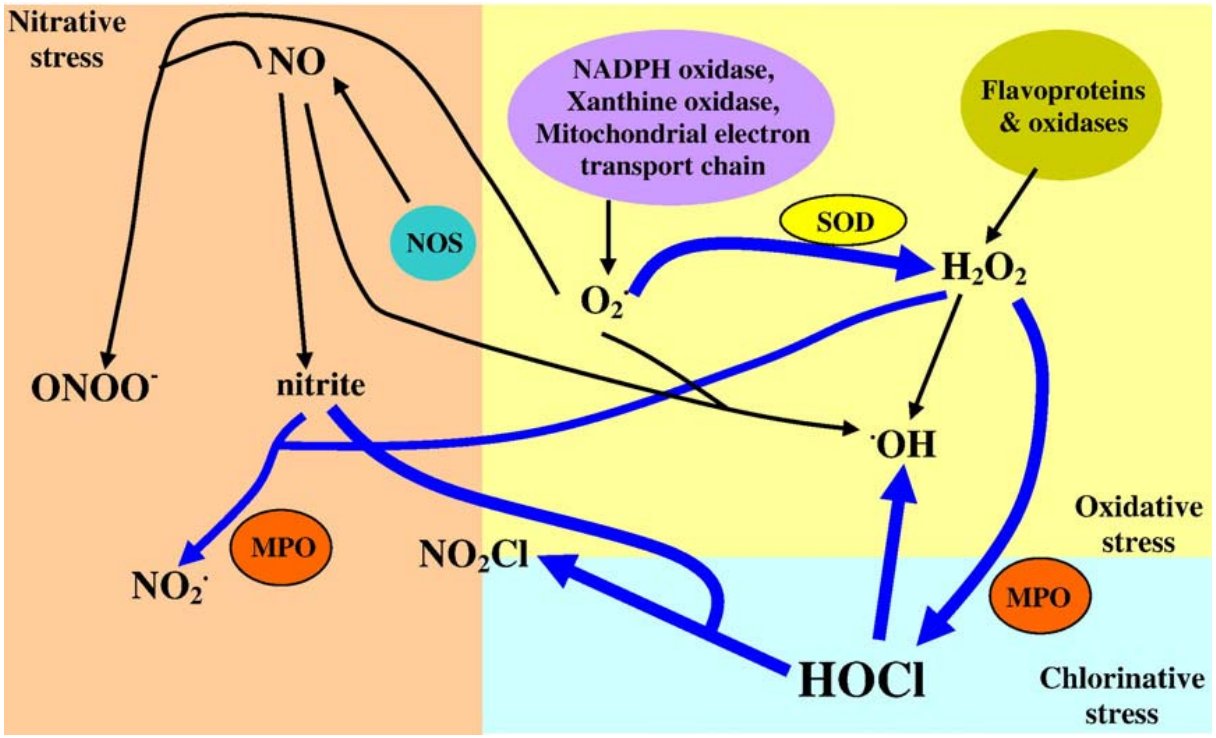

Figure 8: Overview on interactions between different redox-systems producing free radicals that can affect the host tissue by either nitrative, chlorinative or oxidative stress.

"In conclusion, chlorinative stress appears to be the centered mediator of neurodegeneration. Blue arrows represent the most likely route for oxidants production in diseased brains." [190] Figure obtained from: [190]

Indeed, MPO has been detected in microglia[192], which are macrophages located at the central nervous system. Especially in brains derived from patients suffering from Alzheimer's disease, MPO has been shown in activated microglia, associated with hippocampal cells as well as with plaques and tangles[193 cited by 186].

Due to unequal empirical findings, proposing either a protective or a detrimental role of the MPO [-463A] allele, a possible contribution of MPO -463G>A genotype to the development of cognitive decline is discussed more controversially (for detailed review see [186]). According to Pope et al., these unsteady results basically come from statistical bias including different selection criteria, lack of statistical power as well as an unequal gender distribution between groups[194 cited by 186]. As an example, the

author mentioned a Finnish study postulating, that homozygous male carriers of the MPO [-463A] allele show a decreased life expectancy[195 cited by 186]. However, this suggests a depletion of this allele in male persons of advanced age indicating a possible source of statistical bias, for example if ages differ between groups[186].

However, increased cognitive impairment with the MPO [-463A] allele, which again is associated with decreased MPO expression, lead one to assume that this occurs "within a more systemic aspect of the disease" [186].

In case of Alzheimer's disease, neurofibrillary tangles and amyloid plaques are highly affine for negatively charged glycoproteins. Additionally, reduced MPO activity may again lead to reduced defense mechanisms against oxidative stress. Hence, any available MPO could affect tissue of homozygous carriers of the MPO [-463A] allele more heavily, what subsequently leads to promotion of cognitive decline[196 cited by186].

1.13. Exercising, Oxidative Stress and Cognitive Decline

As mentioned before, oxidative stress refers to a state of high concentrations of reactive oxygen species that can modify physiological components as for example proteins and lipids. Because the production of reactive oxygen species increases with O_2 intake, physical exercising results in an up to 20-fold elevation of these radicals[197 cited by 198], suggesting that training increases oxidative stress. Nevertheless, different studies lead to different results[198].
Regarding patients suffering from neurodegenerative diseases, Bloomer et al. showed that patients with Parkinsons' Disease performing short-term resistance training showed reduced oxidative stress markers malon dialdehyde (15%) and H_2O_2 (16%)[199].

However, it can be assumed that chronic exercise may cause increased resistance to reactive species, whereas acute, strenuous exercise may increase oxidative stress[200-204 cited by 198].

In conclusion, the effect of physical exercise on cognitive function still merits further investigation. In a recent meta-analysis of11 randomized controlled trials, Angevaren et al. reported that it is still unknown, whether cognitive functions after physical exercise may improve due to enhanced cardiorespiratory fitness or due to other factors that are not yet elucidated[205].

2. AIMS AND HYPOTHESES

In conclusion, the role of oxidative stress in cognitive decline is not yet completely elucidated. Moreover, cognitive functions may also be affected by physical exercising that in turn alters the amount of reactive oxygen species. Thus, our aim is to investigate the role of myeloperoxidase as a reactive oxygen species producing enzyme in exercising elderly people with respect of their cognitive functions, leading to the following main hypotheses:

- Elevated oxidative stress indicated by high myeloperoxidase concentrations may correlate with lower success in cognitive tests. This may be due to brain damage, atherosclerosis and thrombosis caused by reactive species [187,188 and 189 cited by 186].
- Consequently, these alterations may also be visualized via magnetic resonance imaging resulting in abnormal findings (gliosis, changes in cerebrospinal fluid (CSF) space volume).

- Reduced MPO levels may in turn lead to reduced defense mechanisms against oxidative stress. Hence, even small amounts of myeloperoxidase could cause severe damage to brain tissue as reported by [196 cited by186]. Thus, carriers of the MPO [-463A] allele may be of higher risk regarding impaired cognitive functions.

- Preparation for endurance sport events needs constant training. In a first phase, the increase of O_2 intake will facilitate increased production of reactive oxygen species[198]. Thus, MPO serum concentration may be affected.

- As reported by Vaccarino et al., serum myeloperoxidase is associated with major depressive disorder[206]. Thus, we are expecting an interrelationship between MPO levels/genotype and parameters representing subjective well-being as well as scores reached at self-rating tests assessing depressive disorders.

3. METHODS

3.1. STUDY DESIGN

We prospectively enrolled 113 of 130 participants in a longitudinal cohort study consisting of 56 athletes, which were either marathon runners or bicyclists and a control group of 58 participants, which were matched according to age, sex and years of education. Data has been so far collected at baseline; follow-ups are planned after five and ten years. Thus, this report is limited to discussion of baseline measurements.

Athletes had to meet the following study inclusion criteria:

a) Participation in at least one of the following marathons within the last three years: Wachau Half Marathon (21.5 km), Vienna City Marathon (43 km), Carinthian Marathon (180 km bicycle)
b) At least two hours training per week during the recruitment phase
c) Age over 60 years

The following criteria lead to exclusion of the study:

a) Present or past exposure to neurotoxic compounds
b) If their native language was not German (verbal intelligence tests)
c) Diseases affecting CNS functions: stroke, multiple sclerosis, brain tumors, depression, Alzheimer's disease, epilepsy, Parkinson's disease.
d) Cardiovascular diseases
e) Chronic alcoholism (>60 g daily intake of ethanol or diagnosed history of alcoholism)
f) Unwillingness to give informed consent

The study design has been reported previously [207].

Reporting of the study conforms to STROBE[208] and the broader EQUATOR guidelines[209].

3.2. Participant Recruitment

Atheletes were recruited at appropriate events with help of the organizers (2008 Wachau Half Marathon, Vienna City Marathon, Carinthian Marathon) as well as via personal contacts. Participants within the control group were recruited via advertisements (two in the Austrian Newspaper “Neue Kronenzeitung”, one in the Austrian bicyclist journal “Radsport”) as well as via personal contact.

3.3. Ethical aspects

The study has been approved by the ethics committee of the Medical University of Vienna (EK 401/2005). All participants were informed about the study goals and the study protocol by authorized medical personell. Accordingly, all included test persons gave written informed consent. Moreover, all medical procedures conformed to institutional guidelines.

3.4. Cognitive Testing

Several neurophysiological tests were conducted in order to assess different cognitive functions (see table 2 for a detailed list).

Neurophysiological functions	Neurophysiological Test	Task
Screening of Global Cognitive Functions	MMSE (VNTB/CERAD)	Screening of global cognitive functions (orientation, memory, attention, language, visuo-construction and numeracy)
	Clock Drawing Test (VNTB/CERAD)	Drawing a clock, putting in all the numbers and setting hands at 10 past 11
Premorbid Intelligence	WST (VNTB)	Vocabulary Test
Visuo-Construction	TEVK (VNTB)	Clock-Drawing, Cube-copying, Copying a geometrical figure
	Copying Figures (CERAD)	Copying four geometrical figures
Attention	AKT (VNTB)	Selective cancellation of semi-circles
	WAIS-R Subtest: Digit Symbol Subtest (VNTB)	Transformation of digits into corresponding symbols
	C.I. Symbol Counting Task (VNTB)	Counting quadrates and distinguishing them from other symbols
	TMT-A (VNTB, CERAD)	Connecting numbers from 1 to 25
	NAI Stroop Test (VNTB)	Naming colors facing the challenge that the color of the ink doesn't match the name of the color
	C.I. Interference Test (VNTB)	Substitution of A into B and B into A
Language Functions	Semantic Verbal Fluency Test (VNTB, CERAD)	Naming words from a category within 1 min (animals, supermarket items, tools)
	Phonemic Verbal Fluency Test (VNTB, CERAD)	Naming words beginning with a specific letter within 1 min (b, f, l, s)
	mBNT (VNTB, CERAD)	Naming objects from line drawings
Memory		
Verbal Memory	Verbal Memory Immediate Recall (VNTB, CERAD)	Recalling a wordlist after first presentation
	Verbal Memory Total Recall (VNTB, CERAD)	Recalling a wordlist after repeated presentation (5 resp. 3 times)
	Verbal Memory Delayed Recall (VNTB, CERAD)	Recalling a wordlist after 10–30 min
	Verbal Memory Recognition (VNTB, CERAD)	Distinction between learnt words and distracting words

Figural Memory	Figural Memory Total Recall (CERAD)	Recalling geometrical figures after 10–15 min
Executive Functions		
Planning	NAI Maze Test (VNTB)	Finding the right way out of a maze as fast as possible
Shifting and Task Switching Capabilities	TMT-B (regarding TMT-A) (VNTB, CERAD)	Alternating connection of numbers and letters (1, A, 2, B…)
Nonverbal Fluency	Five Point Test (VNTB)	Drawing different figures in connecting points with straight lines
Semantic Verbal Fluency	Semantic Verbal Fluency Test (VNTB, CERAD)	Naming words from a category within 1 minute (animals, supermarket items, tools)
Phonemic Verbal Fluency	Phonemic Verbal Fluency Test (VNTB, CERAD)	Naming words beginning with a specific letter within 1 min (b, f, l, s)
Interference Capability	NAI Stroop Test (VNTB)	Naming colors facing the challenge that the color of the ink doesn't match the name of the color
	C.I. Interference Test (VNTB)	Substitution of A into B and B into A

Table 2: "*AKT* Alters-Konzentrations-Test, *C.I.* Cerebral Insufficiency Test, *CERAD* Consortium to Establish a Registry for Alzheimer's Disease, *mBNT* modified Boston Naming Test, *MMSE* Mini Mental State Examination, *NAI* Nürnberger-Alters-Inventar, *TEVK* Test zur Erfassung der Visuokonstruktion, *TMT-A* Trail Making Test Version A, *TMT-B* Trail Making Test Version B, *VNTB* Vienna Neuropsychological Test Battery, *VSRT* Verbal Selective Reminding Test, *WAIS-R* Wechsler Adult Intelligence Scale – Revised, *WST* Wortschatztest.". (Table with slight modifications reprinted from [207])

Even mild cognitive impairment could be registered[210-213 cited by 207] by classification of cognitive functions that are commonly affected by dementia (visuo-construction, concentration and attention, language, memory, executive functions) using the *Vienna Neuropsychological Test Battery*[212,213] and the testing recommendations mentioned by the *Consortium to Establish a Registry for Alzheimer's Disease* (CERAD)[214] respectively.

The testing procedures for each participant took approximately 90-110 minutes, starting with the *Mini Mental State Examination* (MMSE)[215] and the *Clock Drawing Test*[216] for assessment of global cognitive functions, followed by drawing and copying tasks according to *Test zur Erfassung der Visuokonstruktion* (TEVK)[217] and the CERAD subtest *Copying*. For evaluation of attention, the following neuropsychological tests were applied: *Alters-Konzentrationstest* (AKT)[218], *Wechsler Adult Intelligence Scale* (WAIS-R) subtest *Digit Symbol Subtest*[219], *Cerebral Insufficiency Test* (C.I.) subtest *Symbol Counting Task*[217], *Trail Making Test A*[220], *Nürnberger-Alters-Inventar* (NAI) subtest *Stroop Test*[221], C.I. subtest *Interference Test*[217]. Moreover, testing of language functions was conducted using semantic and phonemic verbal fluency tests as well as the *modified Boston Naming Test* (mBNT)[214]. Then, verbal and figural memory was tested by the Austrian version of the *Memory Assessment Clinics* (MAC) *Grocery List Selective Reminding Test*[222] termed *Verbal Selective Reminding Test* (VSRT)[217] and the CERAD *Wordlist*[214]. Finally, executive functions were examined using the NAI subtest *Maze Test*[221], the *Trail Making Test B*[220], the *Five Point Test*[223] and again verbal fluency and interference tests.

After this, the participants self-evaluated premorbid intelligence[224], subjective memory functions, physiological and psychological well-beeing[225,226], activites of daily life[227] and depression[228,229] via different self-rating scales and forms, as well as positive health practices using the German version of the *Personal Lifestyle Questionnaire* (PLQ)[230,231].

3.5. Preanalytical Procedures: Sample Collection and Storage

Collection of biological material in the framework of the APSOEM-survey was conducted by the *MedUni Wien Biobank*. This facility has been founded in 2007 as a research project within the *Department for Medical and Chemical Laboratory Diagnosis* at the General Hospital Vienna. However, the MedUni Wien Biobank has so far developed firm expertise in pre-analytic handling of liquid biospecimen such as

whole blood, blood serum, blood plasma containing various anticoagulants and urine as well as protein and nucleic acids extracted from whole blood or peripheral blood mononuclear cells. Currently, the MedUni Wien Biobank arranges pre-analytical and analytical procedures for more than 20 particular clinical trials in and outside the General Hospital Vienna. Due to establishment of a total quality management of the home department including certification to ISO 9001:2008, all procedures occur according to Standard Operating Procedures. For this reason, quality and comparability of biological samples and compliance with privacy protection regulations can be assured at any time.

For this study, blood serum and whole blood was collected in-house and delivered to the Department for Medical and Chemical Laboratory diagnosis via the internal sample porter system. There, the samples and the accompanying datasheets respectively were digitally registered. GBO Vacuette® serum tubes, 10mL (Greiner Bio-One, Kremsmuenster, Austria) were centrifuged for 10 minutes at 3500g and room temperature after an hour-long coagulation delay. Then, five aliquots á 400µL were stored in 750µL Matrix 2D-barcoded tubes (Thermo Fisher Scientific, Hudson, USA) and archived in 96-well latch racks on -80 °C, a standard temperature for long time storage of various human body fluids [232,233].

Whole blood was collected into GBO Vacuette® K_2EDTA tubes, 3mL (Greiner Bio-One, Kremsmuenster, Austria), homogenized by repeated inversion and splitted into three aliquots á 500µL, stored in Matrix 2D-barcoded tubes in 96-well latch ratchs at -80°C as well. All aliquotation steps were conducted on an Olympus OLA 2500 sample allocation system (Olympus Deutschland GesmbH, Hamburg, Germany) with multiple optical sample identification features, fully automated fluid aliquotation robots and simultaneous transfer of sample position data to MOLIS (Sysmex, Norderstedt, Germany) laboratory information system (LIMS). Archiving positions and corresponding 2D-barcodes of anonymous samples were stored in a Microsoft Access database (Microsoft, Redmond, USA), physically divided from LIMS containing participant related data. Linkage between these data bases, which is necessary for proper sample identification upon retrieval of biomaterial, requires

confirmation of the *MedUni Wien Biobank Access Request* form by the clinical conductor of the trial and MedUni Wien Biobank as well [234].

3.6. MPO QUANTIFICATION

Measurement of serum myeloperoxidase concentrations was done by means of enzyme-linked immunosorbent assay (ELISA) [235]. This method is based on a sandwich immunoassay technique, for which antigen specific monoclonal antibodies are coated into each well of twelve separate eight-well strips arranged in a 96-well reaction plate. After application of the respective antigen, followed by several washing steps, a secondary, polyclonal antibody specific for this antigen will be added, binding to the pre-bound antigen. After washing again, a substrate solution has to be administered. This solution undergoes a chemical reaction catalyzed by a functional modification of the secondary antibodies F_c-domain resulting in a change in color. This optical transformation is proportional to the respective antigen concentration and can then be measured on a photospectrometer and compared to a standard curve made from known antigen concentrations.

In our case, a Quantikine® Human MPO Immunoassay kit (R&D Systmes, Inc., Minneapolis, USA) was used [236]. For this, a 400µL blood serum aliquot of each participant was retrieved from MedUni Wien Biobank storage ressource and brought to room temperature. For the following immunoassay, a 50-fold dilution of each sample in *Calibrator Diluent RD6-57* was prepared as suggested by the manufacturer.

A MPO standard curve was prepared from a MPO standard solution provided with the kit yielding the following concentrations: 100ng/mL, 50ng/mL, 25ng/mL, 25ng/mL, 12.5ng/mL, 6.25ng/mL, 3.12ng/mL and 1.56ng/mL.

100µL of each standard and sample, diluted 1:2 with Assay Diluent RD1-62 were applied to the pre-coated reaction wells and incubated covered for two hours shaking at room temperature. After completely discarding the fluid, four washing steps with *Wash Buffer* (400µL each well) were performed. 200µL of *MPO Conjugate*, containing the secondary, polyclonal antibody, were added to each well. After that, the solution was incubated again covered for two hours shaking at room temperature. Then, removal of the liquid and subsequent washing was conducted as described before. After addition of 200µL of *Substrate Solution* to each standard and sample, a 20 minutes light-protected incubation period at room temperature was awaited. Following, the reaction was stopped by administration of 50µL *Stop Solution* resulting in a color shift from blue to yellow. Extinction of standards and samples at 450nm was measured in a Wallac 1420 Victor2™ Multilabel Counter (Perkin Elmer, Waltham, USA) using the Wallac 1420 Victor2™ Workstation V 3.00.0.46 R2 (Perkin Elmer) software.

3.7. MPO -463G>A GENOTYPING

Genotyping of the MPO -463G>A polymorphism was done by means of real time PCR with sequence-specific TaqMan® probes. To evaluate the method, a set of 15 randomly chosen samples was additionally analyzed by restriction fragment length polymorphism analysis. Consequently, equality of each participant's results gained from each method has been assessed.

3.7.1. DNA PREPARATION

DNA was extracted from frozen whole blood semi-automated on a *Corbett X-tractor gene CAS 1820* (Qiagen, Hilden, Germany) using *Macherey Nagel Nucleospin 96-Blood* DNA extraction kits (Macherey Nagel, Dueren, Germany). For this, a 500µL aliquot of each participant was thawed and gently homogenized. Each 200µL were transferred to a single position of a 96-deep-

well plate. The whole blood was diluted with 100µL phosphate buffered saline (Invitrogen, Paisley, UK) to avoid inefficient DNA isolation due to hyperviscous starting material. Subsequently, the deep-well plate was loaded onto the X-tractor gene and the required amount of reaction buffers provided by the *Corbett X-tractor gene application software* was supplied to the predefined slots.

All of the following steps were conducted by the automatically. Firstly, lysis of nucleated blood cells was reached by admixture of 150µL *Lysis Solution* (Buffer BQ1 + Proteinase K, 3+1) followed by a 30 minutes incubation time. Afterwards, 400µL of Lysis Buffer (Buffer BQ1 + Ethanol abs., 1+1) had been added before incubation for five minutes. Then the samples were transferred to a capture plate containing 96 distinct affinity chromatography columns with a DNA-specific solid phase and covered with an overlay of 150µL *Buffer B5.* A 15kPa Vacuum was applied for 5 minutes to facilitate migration of DNA to the solid phase and enable flow of solvated cell debris to an attached waste chamber. After this step, the columns were manually inspected for complete aspiration of the cell lysate. In case of insufficient suction, the column material was penetrated with a thin pin (to avoid spilling of the buffer during the following loading steps because of clogged columns) and the sample's DNA was isolated at one of the following runs. The DNA-binding columns were then washed with 600µL *Buffer BW* applying a 20 kPa vacuum for 3 minutes.

3.7.2. RFLP Analysis

After development of a novel method for in-vitro amplification of deoxyribonucleic acids within a certain length[237], several diagnostic methods evolved from this very technique. One of those methods based upon restriction fragment length polymorphisms (RFLP)[238]. This term refers to a setup of two or more alleles at a distinct genetic locus that vary in their resistance to a specific restriction endonuclease. In our case, transition of guanine to adenine at

position -463 of the *MPO* promoter leads to disruption[239] of a 5'-CCGC-3' [240] motif.

However, the intact sequence would act as a recognition site for *AciI*, a type II restriction endonuclease gathered from *Arthrobacter citreus*. Cleavage of double stranded DNA containing a copy of the said sequence would result in two distinct helices, both with sticky ends, i. e. each helix with a two base extension at the 5'-end resulting from the restriction site. For this, one strand has to be cut between the two cytosines of the 5'-**CC**GC-3' site, whereas the complementary strand undergoes disruption between cytosine and guanine of the respective 3'-GG**CG**-5' motif (please note the highlighted bases).[240]

Again, whether the *AciI* restriction site is present or not, this solely depends on the *MPO* -463G>A genotype. Conversely, detection of the restriction site thus gives information about the underlying genotype. Therefore, a 350 bp fragment[239] was amplified by means of polymerase chain reaction[237]. However, besides *MPO* -463G>A, this fragment contained an invariant *AciI* site as an internal control indicating proper digestion[239]. The possible genotypes and their respective pattern of restriction fragments are shown in table 3.

Genotype	G/G	G/A	A/A
Fragment length	169, 120, 61 bp	289, 169, 120, 61 bp	289, 61 bp

Table 3: The three possible genotypes at position -463 of *MPO* in an individual featuring two copies of chromosome 17 are related to three distinct patterns of restriction fragments, that can be visualized by agarose gel electrophoresis[239].

Polymerase chain reaction was conducted within a total volume 25 µL containing the following compounds:

10 ng template DNA
12.5 µL DreamTaq™ Green PCR Master Mix (Fermentas GmbH, St. Leon-Rot, Germany)
10 pMol primer forward: 5′-CGGTATAGGCACACAATGGTGAG-3’ [239]
10 pMol primer reverse: 5′-GCAATGGTTCAAGCGATTCTTC-3’ [239] (both oligonucleotides: VBC Genomics, Vienna, Austria)
ad 25 µL H_2O sterile (Fermentas GmbH)

Subsequent amplification of the 350 bp-fragment was done applying the following conditions[239] on an Eppendorf Mastercycler® (Eppendorf AG, Hamburg, Germany):

32 cycles of:
DNA denaturing step for 30 sec at 94° C
Primer annealing for 30 sec at 56° C
Strand extension for 30 sec at 72° C

After amplification, 5 µL of each PCR product were digested by 15 units *AciI* [239] restriction enzyme (New England Biolabs, Schwalbach, Germany) within one hour reaction time, thus leading to genotype-specific fragments. These fragments were separated on a 2.5 % agarose gel. For this, 1.5 g agarose was resolved in 60 mL 1x TBE buffer (gathered from a 10x stock: Promega Corporation, Madison, USA) and broiled to boiling point while shaking constantly, until agarose was totally solubilized. After the fluid cooled down to a tepid temperature, 3 µg of ethidium bromide EtBr (equivalent to 3 µL out of a dilution with a concentration of 1ng EtBr per µL ddH_2O) were administered due to its intercalating properties[241] featuring the typical fluorescence spectrum of

EtBr-DNA complexes after absorption of ultraviolet radiation[242]. However, this step allows visualization of DNA fragments of different length represented by separated fluorescent bands after gel electrophoresis.
Subsequently, the tepid fluid polymerized after decanting it into a gel electrophoresis carriage. Before it, a multidentate comb for generation of self-contained DNA loading slots has been placed at the upper end of the carriage. Then, 10 µL of the digested PCR fragments were loaded each on a separate slot. However, on the first slot *MspI* digested plasmid *pBR322* (New England Biolabs) was loaded as a size marker.
Detection and recording of fluorescent EtBr-DNA complex pattern was done using a Polaroid MP4+ instant camera system (Polaroid, Minnetonka, USA) and Polaroid 667 black and white instant films (Polaroid).

3.7.3. TaqMan® Allelic Discrimination

Allelic discrimination using TaqMan® (Applied Biosystems, Rotkreutz, Switzerland) technology is based on the so called 5'-nuclease allelic discrimination assay[243-248 cited by 249]. However, the method uses both, hybridization combined with 5'-nuclease activity as well as fluorescence detection[250]. TaqMan® allelic discrimination can be done on real-time PCR thermocyclers that hence are able to detect changes in fluorescence intensity of different fluorophores[249].
For this, a 100 to 150 bp PCR amplicon containing the bialellic SNP has to be assigned and the respective flanking primers must be designed. Sequence detection itself occurs during annealing phase of the PCR by hybridization of sequence specific MGB-probes on the template amplicon. These probes consist basically of 13 to 25 nucleotides, whereas the variable base lies at the 3'-end (but not within the two most 3' bases). Moreover, the oligonucleotide features a 5'-fluorophor. However, its fluorescence is absorbed by a 3' non fluorescent quencher NFQ. Additionally, at the 3' end a minor grove binder MGB facilitates hybridization with the template thus leading to a possible reduction of probe length. Each PCR setup contains a set of two probes, each

labeled with a discriminative fluorophor, representing either allele of a biallelic SNP. During PCR extension phase, *Taq* polymerase elongates the primer located at the 3'-end of a probe-bound template from its 3'- to its 5'-end. Thereby, the 5' nuclease activity of *Taq* polymerase leads to digestion of the probe's 5'-end and thus releases the fluorophor into solution. That way, its fluorescence is no longer absorbed by the still probe-bound NFQ. The increase of fluorescence detected in real-time by means of fluorometry thus reflects preceding hybridization of either probe 1, probe 2 or in case of heterocygosity both of them and therefore indicates the presence of the respective alleles within the amplicon. Consequently, a scatter plot can be drawn showing each sample's fluorescence in both fluorophores colors, showing three distinctive clusters representing homocygous wild-type, homocygous mutant as well as heterocygous donors. [244]

In our case, real-time PCR was conducted in a 384-well format on an ABI 7900HT Fast Real-time Thermocycler (Appliedbiosystems) in total reaction volumes of 5 µL containing the following compounds:

2.5 µL TaqMan® Genotyping Mastermix (Applied Biosystems)
2 pMol primer forward: 5'-CTTGGGCTGGTAGTGCTAAATTC-3' [251],
2 pMol primer reverse: 5'-GTAATTTTTGTATTTTTCCTTAGGCAAGAAGC-3' [251] (All oligonucleotides: VBC Genomics)
1.5 pMol probe [-463G]: 5'-VIC- TCCACC**C**GCCTCAG-MGBNFQ-3' [251]
1.5 pMol probe [-463A]: 5'-6FAM-TCCACC**T**GCCTCAG-MGBNFQ-3' [251] (All probes: Applied Biosystems)
ad 5µL H_2O sterile (Fermentas GmbH)

Samples were genotyped in duplicate. PCR reaction samples without DNA have been located on various positions of the 384-well PCR-plate (Applied Biosystems) as (cross-) contamination controls.

PCR reaction occurred under the following conditions:

Initial denaturation step for 10 min at 95°C
40 cycles of:
DNA denaturing step for 15 sec at 95° C
Primer annealing/strand extension for 60 sec at 60° C

Then, a scatter plot of fluorescence intensity was generated using SDS 2.3 sequence detection software (Applied Biosystems).

3.8. Statistical Analyses

Metric data showing a normal distribution is given as mean and standard deviation. Other metric data as well as ordinal scaled variables are presented as median and interquartile range. Quantitative data is given as counts and percentages. Correlations of metric variables featuring a normal distribution as well as point-biseral correlations are computed by Pearson's-tests, monotonous relations between elsewise distributed data are assessed using Spearman's-rank-correlation-test. Variables, which means show a normal distribution are compared by one-way ANOVA and T-tests. Intergroup-comparison of non-normal distributed metric and ordinal scaled data is done using Man-Whitney-U tests (in case of two independent groups) and Kruskal-Wallis-tests (in case of more than two independent groups. Distributions of qualitative data are compared using Chi-squared-tests. Significances must be interpreted one-tailed unless indicated otherwise.

All computations are accomplished using IBM SPSS Statistics 19.0 (IBM Corp., New York, USA).

4. RESULTS

4.1. PARTICIPANT CHARACTERISTICS

	Count		**Percentage**	
Female/total participants				
Controls	6/56		10.7%	
Athletes	6/58		10.3%	
	Mean		**SD**	
Age				
Controls	65.88 y		4.53	
Athletes	65.96 y		4.74	
Education				
Controls	11.62 y		4	
Athletes	11.07 y		4.08	
	Median		**IQR**	
Serum MPO level				
Controls	890.89 ng/mL		616.25 ng/mL	
Athletes	902.6 ng/mL		640.79 ng/mL	
Male participants	903.4 ng/mL		656.92 ng/mL	
Female participants	674.45 ng/mL		609.51 ng/mL	
	Count	**Percentage**	**MPO Median**	**MPO IQR**
MPO-463G>A Genotype				
G/G	70	61.9%	915.68 ng/mL	616.15 ng/mL
G/A	38	33.6%		
A/A	5	4.4%	585.5 ng/mL	210.95 ng/mL

Table 4: Overview on participant characteristics

We included 56 marathon runners or bicyclists and 58 participants within the control group, of which 50(athletes)/52(controls) were of male sex. The mean age of the athlete group was 65.96 years (SD=4.74) compared to 65.88 years (SD=4.53) in the control group. Athletes experienced education for 11.07 years (SD=4.08), control participants for 11.62 years (SD=4.0). MPO serum levels (athletes: median=902.6 ng/mL, IQR=640.79 ng/mL; controls: median=890.89 ng/mL, IQR=616.25) did not differ significantly between groups (two-sided p=0.93).

There was no statistically intergroup difference in MPO -463G>A genotype frequencies (athletes: MPO[-463G]/MPO[-463G]=61.8%, MPO[-463G]/MPO[-463A]=34.5 %, MPO[-463A]/MPO[-463A]=3.6 %; controls: MPO[-463G]/MPO[-463G]=62.1%, MPO[-463G]/MPO[-463A]=32.8 %, MPO[-463A]/MPO[-463A]=5.2 %, p=0.92). However, genotype frequencies were in Hardy-Weinberg-equilibrium in both groups (athletes: Chi-squared=0.109, p=0.74; controls: Chi-squared=0.056, p=0.81). Expectedly, serum MPO levels were substantially lower in MPO[-463A] homozygous participants than in persons with heterozygous or MPO[-463G] homozygous genotype (median=585.5 ng/mL, IQR=210.95 ng/mL, compared to median=915.68 ng/mL, IQR=616.15 ng/mL, p<0.01).

MPO plasma levels were significantly higher in male (median=903.4 ng/mL, IQR=656.92 ng/mL) than in female participants (median=674.45 ng/mL, IQR=609.51 ng/mL, p<0.05). MPO-463G>A Genotype distribution in male participants (MPO-463[G]/MPO-463[G]=59.8%, MPO-463[G]/MPO-463[A]=36.3 %, MPO-463[A]/MPO-463[A]=3.9 %) was not statistically different from that in female persons (MPO[-463G]/MPO[-463G]=81.8%, MPO[-463G]/MPO[-463A]=9.1 %, MPO[-463A]/MPO[-463A]=39.1 %, two-sided p=0.17).

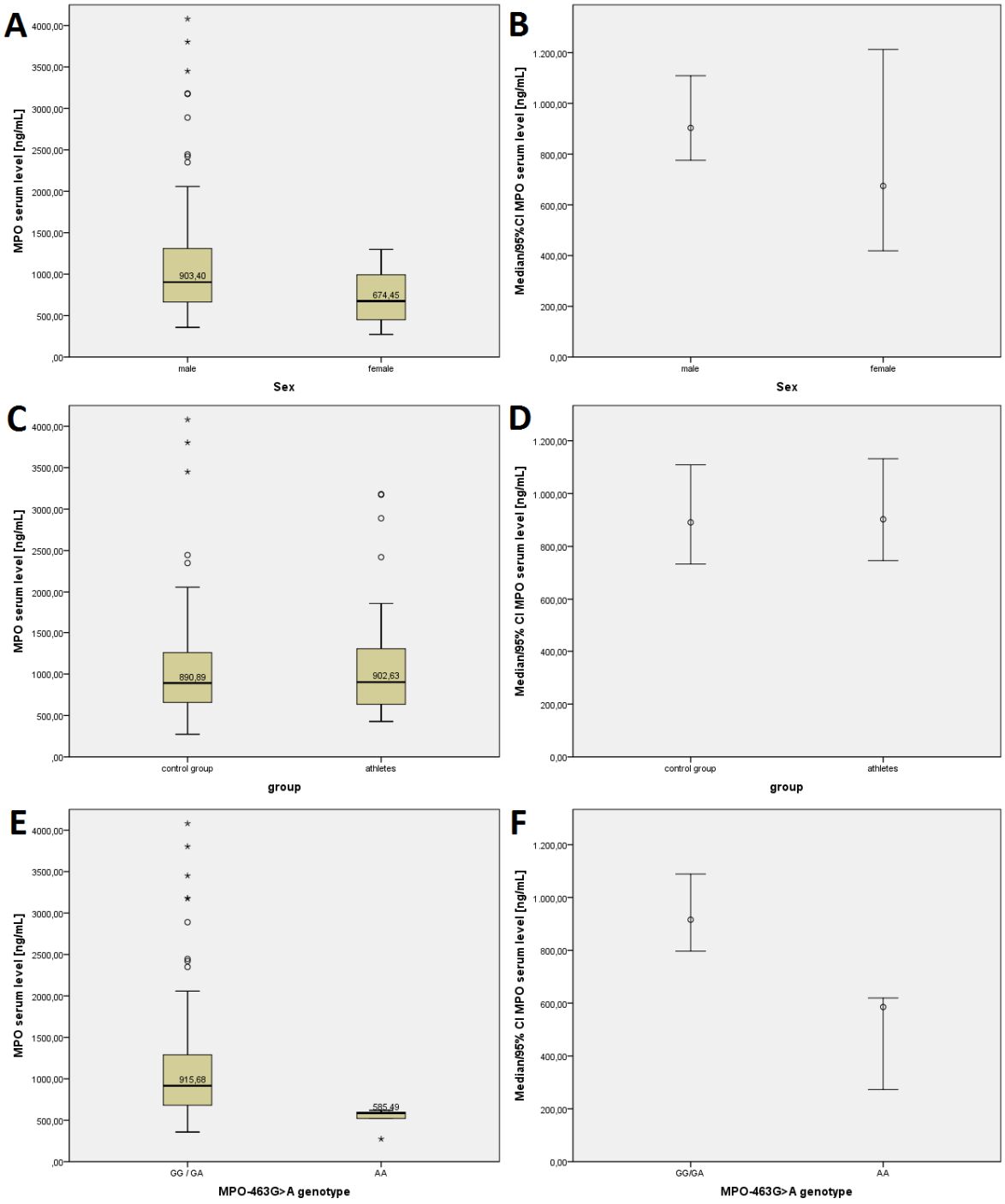

Figure 9: Serum Myeloperoxidase level dependent from sex (A), athleticism (C) and MPO-463G>A genotype (E). Man-Whitney-U tests have been performed for comparison of serum MPO levels in male and female (B)/control participants and athletes (D)/genotype (F), data is presented as mean and 95 % CI.

4.2. MYELOPEROXIDASE AND COGNITIVE TESTS

Please notice that associations between variables showing a normal distribution are given as Spearman's r, whereas correlations between variables with other distributions are presented as Spearman's ρ. MPO serum levels negatively correlate with scores (1-10) from Clock Drawing tests ($\rho=-0.2$, $p<0.05$). In a partial correlation analysis with WST_IQ score, age and sex as control variables, this association persisted in the total study population ($\rho =- 0.214$, $p<0.05$) as well as in the control group ($\rho=-0.328$, $p<0.01$), but not in athletes. Moreover, athletes with higher serum MPO take longer to finish the NAI maze test ($r=0.25$, $p<0.05$). Keeping said control variables constant, the result persisted ($r=0.253$, $p<0.05$). Results in the control group did not reach significance. However, there was no association between MPO level and errors during NAI maze test at all. Accordingly, serum MPO levels did also show a significant partial correlation with the time it took participants to finish Trail Making Test B ($\rho=0.165$, $p<0.05$) as well as with the ratio [time for Trail Making Test B]/[time for Trail Making Test A] ($\rho=0.213$, $p<0.05$). Group-dependently, only athletes showed an association of MPO levels at partial correlation analysis with Trail Making Test ratio ($r=0.391$, $p<0.01$) and Trail Making Test B ($r=0.194$, $p<0.05$).

Interestingly, participants of the control group with higher serum MPO showed less perseverations when they were told to enumerate words starting with the letter "L" at phonemic fluency tests ($r=-0.23$, $p<0.05$). However, these findings did neither show for athletes at bivariate correlation, nor for both groups after partial correlations (said control variables). Furthermore, in MPO[-463A] homozygotes showed a trend for lower WST-IQ ($p=0.054$).

There was no link between cognitive test scores and MPO[-463A]/MPO[-463A] genotype except for a higher amount of perseverations at semantic ($Z=-2.45$, two-sided $p<0.05$) and phonemic fluency tests starting with the letter "l" ($Z=-2.24$, two-sided $p<0.05$) in the control group.

4.3. Myeloperoxidase And Brain Imaging

Participants with increased cerebrospinal fluid space volume featured elevated serum MPO levels (median 1039.8 ng/mL, IQR=628.98 ng/mL compared to median=835.3 ng/mL, SD=634.6, $p<0.05$), unless these findings lost statistical significance in group-wise analysis, although there was a trend for higher serum MPO levels in athletes with CSF space accentuation ($p=0.06$ compared to $p=0.19$ in the control group). In detail, athletes with serum MPO levels >675 ng/mL were more likely to develop CSF space accentuation (20% compared to 45% in participants with lower MPO levels, $p<0.05$) with a relative risk (RR) of 1.33 (95%CI: 0.98 – 1.8).

Furthermore, study participants with subcortical glial modifications had higher serum MPO levels (median=924.06 ng/mL, IQR=862.55 ng/mL) than participants with glial modifications within the central medullary layer (median=746.05 ng/mL, IQR=568.84 ng/mL, $p<0.05$).

There was no association between MPO-463G>A genotype and pathological findings from MR-imaging.

4.4. Myeloperoxidase And Subjective Well-being

Interestingly, we found a statistical association between serum MPO levels, and scores from self-rating questionnaires assessing parameters of subjective well-being. Hence, self-ratings regarding “overall health perception” correlated with serum MPO in athletes by $r=-0.23$, $p<0.05$. In contrast, there were correlations/trends between serum MPO and “vitality” ($\rho=-0.23$, $p<0.05$), “physical pain” ($\rho=-0.21$, $p=0.056$), “overall health perception” ($\rho=-0.2$, $p=0.07$) and “mental well-being” ($\rho=-0.2$, $p=0.07$) in the control group.

Consequently, participants homozygous for the MPO[-463A] awarded significantly more points regarding their “vitality” (Z=-1.65, p<0.05), “mental well-being” (Z=-1.95, p<0.05) and “mental scale” (Z=- 1.87, p<0.05).

Moreover, athletes with high serum MPO (>675 ng/mL) scored significantly higher in Geriatric Depression Scale self-ratings (p<0.05), however, none of the marathon runners reached the test range indicating mild (10-19) or severe (20-30) depression. Furthermore, there is also trend for higher scores in MPO[-463G] homozygotes compared to MPO[-463A] homozygous study participants (Z=-1.481, p=0.088). Interestingly, correlation coefficients (Geriatric Depression Scale score X MPO level) solely reached significance in participants heterozygous for the MPO[-463A] allele (ρ=0.31, p<0.05). In a groupwise comparison, no significant results could be computed. Nevertheless, there was a trend for an association of serum MPO and Geriatric Depression Scale Score in athletic MPO[-463A]-carriers (ρ=0.33, p=0.086).

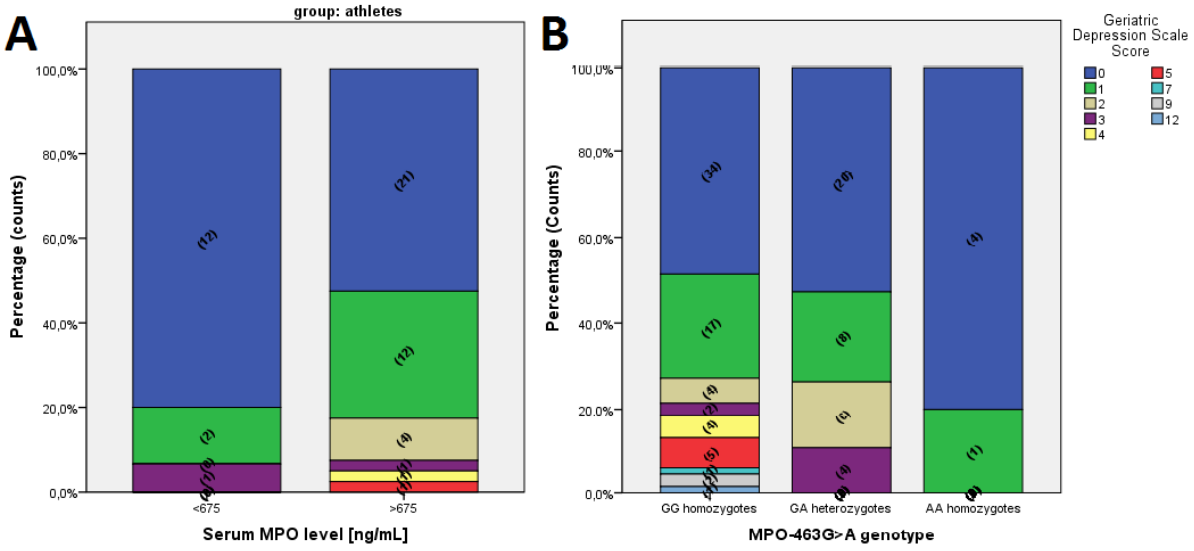

Figure 10: Distribution of Geriatric Depression Scale scores has been compared between athletes with high (>675) and low (<675 ng/mL) serum MPO by Man-Whitney-U test **(A)**. The same was done for MPO[-463G] and MPO[-463A] homozygotes **(B)**. Scores of the Geriatric Depression Scale can be interpreted as follows: 0-9=no depressive disorder indicated, 10-19=mild depressive disorder indicated, 20-30=major depressive disorder indicated. Numbers in brackets indicate absolute counts.

5. DISCUSSION

5.1. MYELOPEROXIDASE AND PATIENT CHARACTERISTICS

Myeloperoxidase levels did not differ significantly in atheltes and controls. A study conducted by Garatachea et al.,[252] MPO levels of kayakers at different stages of training have been measured. Interestingly, the investigators found no significant associations between MPO concentrations and intensity of the training. Our findings moreover conform to previous publications, which indicate, that plasma MPO levels may be elevated immediately after exhaustive training, but return to their previous concentrations at rest[253-255].

5.2. MYELOPEROXIDASE AND COGNITIVE TESTS

Interactions between MPO serum level and cognitive test outcome could be shown for assessments of global cognitive functions, as well as for executive functions. On the one hand, we found significant correlations between plasma MPO and worse performance in clock drawing test, NAI maze test and Trail-Making test B. On the other hand, participants homozygous for the MPO[-463A] allele, which thus feature low serum MPO were more likely to repeat said words when they were asked to mention as many words as possible starting with the letter “L” (phonemic fluency) or belonging to a certain group (semantic fluency) within one minute of time.

As mentioned before, oxidative stress may contribute to cognitive impairment by either increased atherosclerosis and thrombosis[187,188] or selective brain damage[189]. Moreover, there is evidence that strenuous exercising in contrast to moderate physical activity enhances production of reactive oxygen species and thus causes oxidative stress[198]. This hypothesis has been supported by findings from animal experiments, as mice showed worse performances in tests of cognitive function after

exhaustive exercising[256]. Consequently, athletes with higher basal MPO levels may experience harmful MPO concentrations after intense exercising. Indeed, our findings support this hypothesis. Athletes with lower serum MPO scored better in NAI maze test as well as in Trail-Making test B/A ratio and in Trail Making Test B, all of them representing executive functions (planning and task shifting capabilities respectively). There again, partial correlations between the clock drawing test assessing global cognitive functions could only be evinced significantly in the control group, indicating that global cognitive functions could be protected by endurance exercising. Since assessment of global cognitive function includes analysis of a variety of different mechanisms and aspects of cognition, executive cognitive functions represent a specific cognitive domain, which involve development and performance of purposive behavior, as well as judgment and abstract thinking[257 cited by 258]. As shown before, executive impairment directly influences instrumental activities of daily livingand may thus be a stronger predictor of functional difficulty than global cognitive functions[257,259,260].

To the best of our knowledge, a connection between low serum MPO level/homozygous MPO[-463A] genotype and increased perseverations in semantic/phonemic fluency test has not been reported before. Perseveration scores of fluency tests are a sensitive marker for frontal dysfunction[261]. Consequently, a higher amount of repetitions in A/A homozygous participants could indicate frontal dysfunction due to less protective defense mechanisms [196 cited by186].

5.3. Myeloperoxidase and Brain Imaging

As mentioned before, microglia cells, which build up the cell mediated immune system of the central nervous system, express moderate amounts of MPO[192]. Hence, myeloperoxidase can leak from activated microglia to the intercellular space. There, the enzyme could cause damage to cerebral host tissue via production of ROS.

Truly, we could find a statistical association between attenuation of cerebrospinal space as a sign of cerebral atrophy and MPO serum levels. However, in groupwise partial correlations, these – statistically non-significant – result was more prominent in athletes than in control participants (p=0.06 compared to p=0.19). Comparing athletes with serum MPO levels <675 ng/mL to athletes with lower MPO concentrations, the first had a slightly elevated relative risk (1.33) of developing CSF space accentuations. These findings again indicate, that high MPO levels could be more detrimental in endurance athletes than in the average population.

5.4. Myeloperoxidase and Subjective Well-being

In our case, Myeloperoxidase levels are associated with self-rating scores regarding the state of health and mood. In fact, Vaccarino et al. reported elevated serum MPO in patients suffering from major depressive disorder[206]. This again supports our hypothesis, that serum MPO levels are associated with mood and mental disorders. Again, we showed a significant association between high Geriatric Depression Scale scores and homozygosity for the MPO[-463G] allele. Galecki et al. observed, that the MPO[-463G] allele increased the risk of suffering from a depressive disorder by 1.5 (95% CI: 1.05 – 2.33), whereas the risk for MPO[-463G] homozygous participants was increased 1.7-fold (95% CI: 1.09 – 2.79)[262].

Interestingly, the association of serum MPO and Geriatric Depression Scale score is significant in carriers of the MPO[-463A] allele, but not in participants homozygous for the more common MPO[-463G] allele. This implies that there may be a loss of defense mechanisms due to decreased myeloperoxidase expression as mentioned before. In this case, higher amounts of myeloperoxidase could cause more severe damage than in tissue featuring more adequate host protection against oxidative stress[196 cited by186].

5.5. Limitations and Benefits of This Study

However, this is not a final report of the APSOEM-trial. Much more it must be seen as a status record of what we so far learnt about what myeloperoxidase serum level and genotype may contribute to cognitive function, alternations in the physical constitution of the brain, as well as subjective well-being and mood state in elderly marathon runners and bicyclist. Having passed a first and second follow-up after five and ten years respectively, we will be able to study time dependent variables. Then it will be possible to investigate, whether there is a difference, how high serum MPO influences the onset time of more manifest cognitive decline, brain atrophy and mental parameters in athletes and non-athletes.

We have to disclose as a limitation of our study, that there are several discrepancies regarding the recommended biomaterial for MPO level measurement. Although the manufacturer's protocol of the used ELISA-kit suggests cell culture supernates, cell lysates, plasma, saliva, urine, human milk and of course also serum as adequate material[236], Shih et al.[263] refer to Chang et al.[264], when they reported, that blood coagulation MPO is spilled by leukocytes and should thus not be used for measurement of myeloperoxidase concentrations. By this mechanism, MPO concentrations increase with time until separation of cells and serum. Nevertheless, they stated an acceptable correlation between their recommended material EDTA-anticoagulated plasma and serum (r=0.821, slope=4.14, intercept=33.29). Again it should be said, that because of standardized preanalytical procedures including throughput-time, processing and storage of samples, the disadvantages of the used material might be mitigated to the greatest possible extend.

Another limitation affecting computations on possible genotype-dependent variables is the relative small sample count resulting in a small number of participants being homozygous for the minor allele. Thus, associations of the MPO[-463A]/MPO[-463A] genotype should be interpreted with caution. Assuming that we wanted to estimate the required sample size for calculation of differences in Mann-Whitney-U-tests with

a large effect size (d=0.5) presuming a statistical power of 0.8 (1 – β-error) and an α-error of 0.05, the statistics tool G*Power[265,266] would suggest to analyze 28 MPO[-463A] homozygous participants which means that we had to collect at least 648 persons (presuming 4% of the study population homozygous for the minor allele).

Again, we are aware that because of statistical correlation, although significant, causal association of different variables cannot be assumed. However, by partial correlation, potential influences of other variables could be kept constant. After follow up, it will be possible to assess by multivariate regression analysis, whether myeloperoxidase serum level and genotype respectively could be used as an independent risk marker for cognitive decline in elderly sportspeople.

To the best of our knowledge, this is the first prospective cohort study on the effect of myeloperoxidase and thus oxidative stress on cognitive function in a cohort of elderly endurance athletes.

Concludingly, we suggest that myeloperoxidase affects executive function, morphological cerebral degeneration and subjective well-being dependent from physical activity and MPO-463G>A genotype as well. However, there is only little knowledge about the underlying mechanisms, which thus still have to be elucidated.

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7. CURRICULUM VITAE

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Schulbildung

1996-2004	BRG mit naturwiss. Schwerpunkt, Spittal/Drau, Ktn. Maturiert mit ausgezeichnetem Erfolg.
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1991-1992	Vorschule Reisseck/Mölltal

Universitäre Ausbildung

2008-laufend	Studium der Humanmedizin an der Medizinischen Universität Wien. Ergebnis des Eignungstests: unter den besten 5 %.
2006-laufend	Studium der Politikwissenschaft an der Universität Wien.
2005-laufend	Studium der Molekularen Biologie an der Universität Wien. Absolvieren des Auswahlverfahrens als 2. der Rangliste.

Wissenschaftliche Praktika (Wahlbeispiele)

2008	Prof. Ines Swoboda, Christian-Doppler-Labor für Allergieforschung, Institut für Pathophysiologie, MedUni Wien (Construction of recombinant pET17b containing Ole e 9)
2010	Prof. Raute Sunder-Plassmann, Klinisches Institut für Medizinische und Chemische Labordiagnostik, MedUni Wien (Expression of wild type and 121Q mutant (rs1044498) human plasma cell glycogprotein-1/PC-1 in HEK293 cell line)
2011	Dr. Thomas Perkmann, Klinisches Institut für Labormedizin, MedUni Wien (Plasma Myeloperoxidase level and MPO genotype: useful markers for prediction of MACE in male and smoking patients suffering from PAD)

Berufliche Laufbahn

2007-laufend	Stv. Leitung und QM-Koordinator der MedUni Wien Biobank
2009	Dr. Müller Marktforschung
2008-2009	Tutor an der Universität Wien, Inst. für Biochemie
2006	Eventpromotion Mediaprint AG
2006	Ferialpraktikum Österr. Post AG, Schalterdienst
2004-2005	Rettungssanitäter (Zivildienst), RK St. Johann i. T.
2003	Ferialpraktikum im Gastronomiebereich

Sonstige Tätigkeiten

2009-2011	Vorsitzender der ÖH-FV Molekulare Biologie an der Uni Wien
2007-2011	Vorsitzender der ÖH-StV Molekulare Biologie an der UniWien

Skills und Kenntnisse

2010-laufend	Ausbildung zum Trainer in polit. Rhetorik nach ASG
2010-laufend	Interner Auditor ISO 9000 in Ausbildung (GALP)

2010-laufend	SPSS Statistikapplikation (fundierte Anwenderkenntnisse)
2010	QM-Beauftragter (GALP)
2009	Gefahrengutbeförderung (VAMED)
2004	Rettungssanitäter (Österreichisches Rotes Kreuz)
2004	European Computer Driving License
2003	Rhetorik und Präsentation (u.a. Institut „The Training“)

Fremdsprachen

Deutsch (Muttersprache)
Englisch (Wort und Schrift)
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Wissenschaftliche Mitgliedschaften

Österreichische Gesellschaft für Molekulare Biowissenschaften und Biotechnologie
Bund Sozialdemokratischer AkademikerInnen

Kongress- und Tagungsbesuche

2010	„Summer Institute: Genetics, Ethics and Clinical Translation”, Egmond aan Zee, NL
2008	Member of „Arbeitsgruppe zur Errichtung einer nationalen Biobank des BMWF“ , Graz, A
2008	“ESF BIOBANKS 2008”, St. Filou, ESP
2008	“Danubian Biobank Consortium: Meeting on Work Package 5: Scientific Strategy. Modul 1: Transdifferentiation and Pathomechanisms of Organ Dysfunction in the Metabolic Syndrome Complex”, Bratislava, SK
2008	“Danubian Biobank Consortium: Genetic background of Chronic Renal Insufficiency and Kidney transplantation”, Vienna, A
2007	„Gemeinsamer Kongress der ÖGLMKC und der DGKL“ , Vienna, A
2007	„2. genXpress RealTime PCR/Corbett User Meeting “, Wr. Neudorf, A

Poster und Vorträge

1. **Haslacher, H**, Perkmann, T, Gruenewald, J, Exner, M, Endler, G, Scheichenberger, V, Wagner, O & Schillinger, M: Plasma myeloperoxidase and peripheral arterial disease. *KILM Institutsseminar*, 2011, Talk.
2. Slavka, G, Perkmann, T, **Haslacher, H**, Wagner, O & Endler, G: Mean platelet volume is a predictive parameter for overall vascular mortality and ischemic heart disease. *Clin Chem Lab Med,* 47**:** Poster: 220, 2009.
3. Perkmann T, Endler, G, **Haslacher, H**, Rumpold, H, Slavka, G, Wagner, O, Exner, M, Minar, E & Schillinger, M: Plasma Myeloperioxidase (MPO) as an independent risk marker for major cardiovascular events in patients with peripheral artery disease. *Clin Chem Lab Med,* 47**:** Poster: 135, 2009.
4. **Haslacher, H**, Perkmann T, Endler, G, Rumpold, H, Slavka, G, Wagner, O, Exner, M, Minar, E & Schillinger, M: Plasma Myeloperioxidase (MPO) as an independent risk marker for major cardiovascular events in patients with peripheral artery disease. *Abbott Myeloperoxidase Investigator Meeting.* Frankfurt/Neu Isenburg, 2009, Präsentation in absenz.
5. Aschauer, S, Mittermayer, F, Wagner, C, Schmidt, W, Brunner, M, **Haslacher, H**, Wolzt, M & Müller, M: Association of a genetic variation on chromosome 9p21.3 with reduced arterial vasodilator function in healthy volunteers . *Basic & Clinical Pharmacology & Toxicology,* 105**:** Talk: 21, 2009.
6. **Haslacher, H**, Perkmann, T, Ponweiser, E, Dellago, S & O, W: Preanalytic factors influencing the quality of mRNA preparations from human PBMCs. *ESF BIOBANKS 2008.* St. Filou (ESP), Poster, 2008.
7. **Haslacher, H**: The AKH Biobank initiative. *Danubian Biobank Consortium: Genetic background of Chronic Renal Insuffieciency and Kidney tranplantation*. Wien, 2009, Talk.
8. Kampitsch, T, **Haslacher, H**, Hojak, A, Reichhold, I, Marsik, C, Endler, G & Wagner, O: Evaluation of a semi automated sample storage and archiving system based on the Olympus PLA 2500. *Clin Chem Lab Med,* 45**:** Poster: A103, 2007.
9. **Haslacher, H**, Kampitsch, T, Hojak, A, Marsik, C, Endler, G & Wagner, O: The AKH biobank initiative – bridging the gap between freedom of academic

research and protection of privacy. *Clin Chem Lab Med,* 45**:** Poster: A103, 2007.

Publikationen

1. ***Haslacher, H,*** Perkmann, T, Lukas, I, Barth, A, Ponocny, E, Michlmayer, M, Scheichenberger, V, Wager, O & Winker, R: Serum myeloperoxidase level and cognitive function in elderly marathon runners. (*In Submission*), 2011.
2. ***Haslacher, H***, Perkmann, T, Gruenewald, J, Exner, M, Endler, G, Scheichenberger, V, Wagner, O & Schillinger, M: Plasma myeloperoxidase and peripheral arterial disease. *Eur J Clin Invest* (*in press),* 2011.
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