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Investigations on the plasma membrane electron transfer activity in human erythrocytes

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# Abstract

The particular use of intracellular reduction equivalents to reduce substrates in the extracellular environment by employing plasma-membrane-associated enzymes and electron carriers is common to effectively all eucaryotic cells. It is referred to as transplasma membrane electron transport (PMET) and mainly serves to protect cells and surrounding tissues from exogenous damages due to reactive oxygen species and free radicals.

The present work aimed to characterise the PMET activity of human red blood cells (RBCs) from distinct perspectives. The main focus herein was on investigating differences in PMET activities between genders, but we also performed analyses which concerned the activity response of RBCs to conditions like increased osmolaric stress, excess glucose or phosphatase inhibiton. Furthermore, we tried to identify age-related NADH oxidases (arNOX) - superoxide-generating components of the PMET system already detected in human body fluids - in RBCs of aged subjects by immunoblottings and reduction activity assays based on cytochrome c and superoxide dismutase. PMET activities were determined spectrophotometrically by monitoring the extracellular reduction of ferricyanide and ferricytochrome c, whereby the use of the latter for this purpose was introduced and established for the first time and allowed us to define reductive activities more precisely.

The gender-specific analysis revealed that redox activities of male and female subjects significantly differed in several aspects, indicating that the PMET system is regulated in a gender-dependent manner. Our preliminary observations regarding cell treatment procedures were mostly not clearly classifiable yet but could provide indications insofar, as hyperosmolaric stress tended to increase PMET activity, whereas the phosphatase inhibitor rather decreased it. Concerning arNOX, our results could not support the hypothesis that this oxidase exists in human RBCs.

In conclusion, we could show that the plasma membrane redox system in human erythrocytes is subject to variations, which are influenced by gender-specific factors, a finding, which importantly benefited from the ferricytochrome c based, yet unpublished method we established.

# Table of contents

1 Introduction 1

1.1 Plasma membrane electron transport systems 1

1.1.1 The methodical use of physiological and non-physiological electron donors and acceptors 3

1.1.2 Insights into key components of the transplasma membrane redox system 4

1.2 ENOX protein family 8

1.2.1 The functional roles of ENOX proteins 10

1.2.2 Age-related NADH oxidases 12

*1.2.2.1 arNOX and the TM9 superfamily of transmembrane proteins* 13

1.3 Aim of the project 15

2 Material and Methods 16

2.1 Buffers and solutions 16

2.2 Blood donations and storage 19

2.3 arNOX activity and protein analysis 20

2.3.1 Red blood cell isolation 20

2.3.2 Lysis and membrane preparation 20

2.3.3 Western blot analysis 21

2.3.4 arNOX activity measurement 21

2.4 Analysing the plasma membrane redox system in erythrocytes 22

2.4.1 Preparation of red blood cells 22

2.4.2 Measuring electron transport across the red blood cell plasma membrane 23

*2.4.2.1 Ferricyanide reduction assay* 23

*2.4.2.2 Cytochrome c reduction assay* 25

2.4.3 Preparation and activity analysis of haemolysates 25

2.4.4 Pretreatment procedures of red blood cells 26

2.4.5 Statistical analysis 28

3 Results 29

3.1 Establishment of a workable method for the reliable determination of ferricytochrome c reduction activity in erythrocytes 29

3.2 Determinations on the possible existence of arNOX in human red blood cells 32

3.2.1 arNOX protein analysis 33

3.2.2 Analysis of erythrocytes for the presence of arNOX activity 34

3.3 The plasma membrane redox system in erythrocytes 36

3.3.1 Gender-related differences in the reductive activities of red blood cells 36

3.3.2 The influence of selected treatment procedures on the plasma membrane electron transport activity of erythrocytes 41

*3.3.2.1 Hyperosmolarity* 41

*3.3.2.2 Hyperglycaemia* 42

*3.3.2.3 Cyclodextrin* 42

*3.3.2.4 Vanadate* 44

*3.3.2.5 Coenzyme $Q_{10}$* 46

3.3.3 Storage-dependent variations in the plasma membrane electron transport of erythrocytes 47

4 Discussion 49

4.1 arNOX was not detectable in whole cell and plasma membrane preparations of human erythrocytes 49

4.2 Gender, hormones and their impact on the reductive potential of erythrocyte plasma membranes 51

4.3 The transplasma membrane redox system of red bood cells is modulated in response to varying conditions 54

5 Conclusion 60

6 References 61

Appendix 75

# 1 Introduction

## 1.1 Plasma membrane electron transport systems

Effectively all cells, independent of tissue or species, possess a complex redox system, referred to as plasma membrane electron transport (PMET), which is characterised by the directed transfer of electrons through the lipid bilayer from intracellular donors onto extracellular acceptors. It is implicated in an array of cellular functions, most essentially contributing to the redox homoeostasis and antioxidative capacitiy of the cell itself and its environment (Rodriguez-Aguilera *et al.* 2000). Living in an oxygenised world naturally implies that cells are constantly exposed to oxidative conditions faciliating the arising of free radicals (Davies 1995). To prevent undesireable alterations of proteins and lipids by oxidation, cells are permanently challenged to keep the amount of oxidative molecules as low as required for intact function. The PMET is suggested to represent a potent redox system enabling cells to adapt to changes in extracellular oxidative stress by transferring reducing equivalents outwards across the membrane (Kehrer and Lund 1994; Villalba and Navas 2000). This is seen as an active process, catalysed by plasma-membrane-associated and -embedded enzymes and involves NAD(P)H and ascorbate/ascorbyl free radicals as intracellular electron donors as well as intramembranous ubiquinones as electron shuttles through the lipid bilayer (Del Principe *et al.* 2011). The importance of that specialised redox system becomes particularly evident during aging and the emergence of several diseases, both processes, in which oxidative stress is thought to be crucially involved (Colavitti and Finkel 2005; Valko *et al.* 2007). However, two components of the PMET system are known to produce superoxide themselves by catalysing the reduction of extracellular molecular oxygen (both will be described in more detail here). Whereas the one, members of the Nox family of NADPH oxidases, is mostly associated with cell signalling processes, the other, termed age-related NADH oxidases, was found to accumulate in plasma membranes of

aged individuals, where it significantly added to aging-related cellular defects (BEDARD and KRAUSE 2007; MORRE *et al.* 2008b). An effectively regulated PMET, thus, is proposed to be an essential prerequisite contributing to healthy aging and longevity (HYUN *et al.* 2006; RIZVI *et al.* 2011).

The transplasma membrane redox activity in erythrocytes is devoted particular relevance to. Erythrocytes as the body's oxygen reserve are especially prone to oxidative damages and hence are assumed to have evolved specific mechanisms to counter them (CIMEN 2008). Indeed, they seem to possess a unique property which is yet not described for other cells and allows them to regulate intracellular and extracellular concentrations of the antioxidant ascorbate (MAY *et al.* 1995b). This mechanism is referred to as ascorbate recycling and seems to exist at least in human erythrocytes (MAY *et al.* 2004; RIZVI *et al.* 2009).

Ascorbate (vitamine C) is an important cofactor for diverse essential enzymatic reactions and a potent source for cellular antioxidants (ARRIGONI and DE TULLIO 2002). Whereas most species have evolutionary conserved mechanisms to synthesise vitamine C, this property was lost in higher primates, including humans, and some other vertebrates, which hence are dependent on a life-long assimilation of that vitamine by food intake (BANHEGYI *et al.* 1997). It is believed that parts of the electrons transferred through erythrocyte plasma membranes are used to regenerate extracellular pools of the antioxidant by reducing oxidised ascorbate (MAY *et al.* 1995b).

In detail, dehydroascorbate (DHA) as the inactive form of ascorbate is imported by GLUT1, a transmembranous protein which usually mediates the uptake of glucose (WANN *et al.* 2006). Inside erythrocytes, DHA gets reduced back to ascorbate, which in turn serves as the major electron donor for the PMET-system-driven extracellular regeneration of ascorbate from its oxidised forms (MAY *et al.* 2001; MAY *et al.* 1995a). Transmembranous duodenal cytochromes b are proposed to be the likeliest redox enzymes involved therein (SU *et al.* 2006). It is strongly suggested that red blood cells thereby play a fundamental role in preserving the levels of ascorbate in blood serum and thus impart oxidative protection to adjacent tissues (MAY *et al.* 1995b; MENDIRATTA *et al.* 1998).

### 1.1.1 The methodical use of physiological and non-physiological electron donors and acceptors

Cells transfer electrons to reduce extracellular substrates, so most methods apply non-toxic, cell-impermeable oxidants, which are spectrophotometrically monitored for their reduction. To allow a more precisely characterisation of transmembrane reducing systems, cell membrane vesicles are generated to contain potential physiological electron donors (ASKERLUND and LARSSON 1991; GREBING *et al.* 1984). The thereby observed activities are defined according to the electron acceptors and donors used and hence are designated donor-acceptor oxidoreductases. As there are numerous compounds utilised for this purpose - particularly for electron acceptors - this overview will address only the most common ones.

Amongst them, ferricyanide, a complex of trivalent iron with six cyanide ligands, is a very established electron acceptor to determine PMET activity (CLARK *et al.* 1981). The reduction of the ferric iron ion in its centre generates ferrocyanide, and this reaction is conventionally measured in two different ways. One possibility is to record the decrease in absorbance of ferricyanide at 420 nm to indirectly determine the amount of ferrocyanide produced (SUN *et al.* 1992). A more popular and quite more sensitive method is the direct measurement of ferrocyanide by the chromogenic chelator bathophenanthroline (4,7-diphenyl-1,10-phenanthroline) (AVRON and SHAVIT 1963). The compound specifically binds and chelates divalent iron ions and thereby significantly increases the absorption of ferrocyanide, which then can be read at 535 nm (CLULEY and NEWMAN 1963).

Other artificial extracellular oxidants are tetrazolium salts, which, when reduced, form soluble and insoluble chromogens called formazans (BERRIDGE *et al.* 2005). They are sometimes added together with intermediate electron acceptors to improve their reduction by cellular transferred electrons. WST-1 for example is frequently used to assess NAD(P)H-dependent transmembrane redox activities (BERRIDGE and TAN 2000; GRAY *et al.* 2011).

The use of NADH as electron donors provides a more authenthic, physiological approach for investigating PMET systems. It is a natural constituent of cytosols and other cellular compartments, serving as a basic reductant for diverse cellular redox processes (YING 2008). NADH is either added to purified enzyme preparations or used to load plasma membrane vesicles (ASKERLUND and LARSSON 1991; LUSTER and BUCKHOUT 1989). In conjunction with potential electron acceptors, it allows the characterisation of plasma-membrane-associated NADH-dependent reductive activities (BERCZI *et al.* 1995; VILLALBA *et al.* 1995).

Ascorbate (vitamine C) in all its redox states also represents a valuable tool to reproduce naturally present conditions for examining PMET (GOLDENBERG *et al.* 2000). In addition, the reduced form of ascorbate, monodehydroascorbate or ascorbyl free radical (AFR), is used twice, once as extracellular electron acceptor and once as intracellular electron donor, because it has the dual property to be either reduced to ascorbate or oxidised to dehydroascorbate (DHA) (HOREMANS *et al.* 1994; VANDUIJN *et al.* 2000). Cells can also be loaded with DHA, which intracellularly (so far only in erythrocytes) gets re-reduced to ascorbate, a potent electron donor to assay transmembranous reductive activities (MAY *et al.* 2001).

### 1.1.2 Insights into key components of the transplasma membrane redox system

Though its existence has already been known for nearly 90 years, it was a long time in coming to identify some of the key players implicated in the transfer of electrons through plasma membranes (figure 1) (VOEGTLIN *et al.* 1924). Among the most extensively investigated enzymes described to participate in PMET, ***NADPH oxidases (Nox)*** are to be mentioned (CROSS and SEGAL 2004). They constitute a protein family of at least seven members and are transmembrane oxidases, generating superoxide at the cell surface in a NADPH-dependent manner (BEDARD and KRAUSE 2007). The enzymes

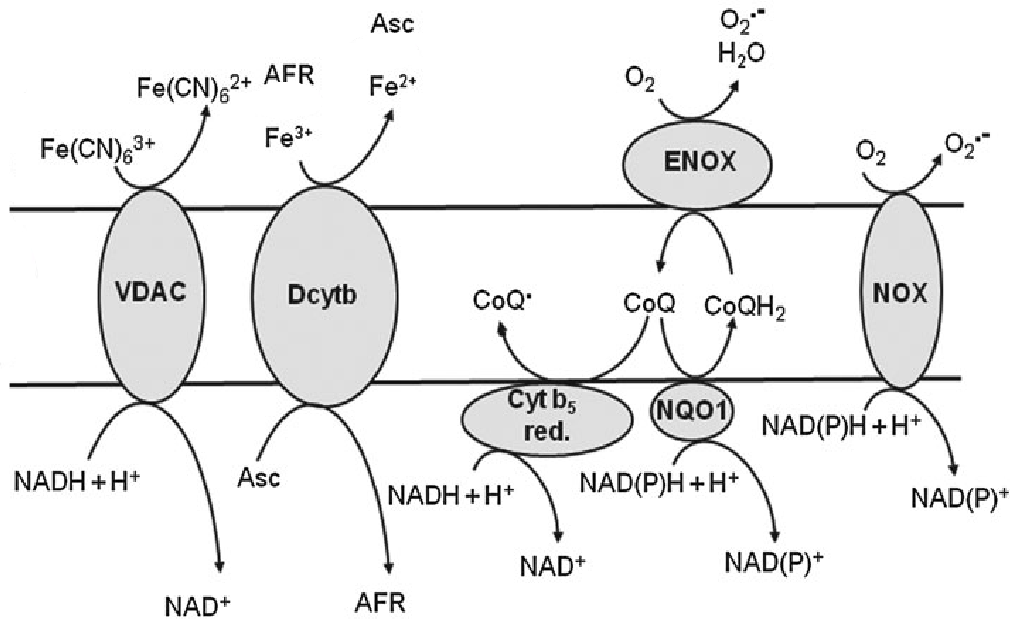


**Figure 1: Key players of the plasma membrane electron transport system.** By now, six enzymes with distinct activities were identified to participate in the transmembranous transfer of electrons. They are either spanning the lipid bilayer or are associated at the internal or external surface and catalyse the reduction of extracellular oxidants at the expense of intramembranous and -cellular reduction equivalents. VDAC = voltage-dependent anion-selective channel; Dcytb = duodenal cytochrome b; Cyt $b_5$ red. = cytochrome $b_5$ reductase; NQO1 = NAD(P)H-ubiquinone oxidoreductase; ENOX = ECTO-NADH oxidase; NOX = NADPH oxidase; Asc = ascorbate; AFR = ascorbyl free radical; CoQ = ubiquinone; $CoQH_2$ = ubiquinol; $Fe(CN)_6$ = ferri- and ferrocyanide, respectively. Illustration was taken from „Trans-plasma membrane electron transport in mammals: functional significance in health and disease“ (DEL PRINCIPE *et al.* 2011)

differ in tissue-specifity and physiological functions, which are yet not fully clarified but mainly base on signal transduction (BROWN and GRIENDLING 2009). The best studied member of the enzyme family, Nox2, is primarily expressed in phagocytic cells, where it is activated to kill invading microbes (RADA *et al.* 2008). Roles in cellular immune defence and inflammatory response are suggested for most Nox proteins, however, they seem to be involved in many other cellular processes (RADA and LETO 2008). Several studies are indicating that Nox-generated superoxide acts as a messenger molecule, initiating and propagating signals for the regulation of gene expression, ion-channels, cell growth and death (BEDARD and KRAUSE 2007; BROWN and GRIENDLING 2009). On the other hand, dysregulated activities of these enzymes can have deletirious effects on cells, as

elevated levels of superoxide are associated with diverse pathologies and aging (OLIVEIRA *et al.* 2010). For example, the aggressive growth of tumor cells and an increased risk for atherosclerosis were reported to be promoted by NOX proteins (GRAY *et al.* 2013; VAQUERO *et al.* 2004)

The formation of superoxide by the reduction of molecular oxygen is also a characteristic property of ***ECTO-NADH oxidases,*** which are located at the cell surface. Their specific activity and cellular functions will be discussed in the next chapter and thus are not further elaborated here.

***NQO1 (NAD(P)H-ubiquinone oxidoreductases)*** are cytosolic proteins mediating the reduction of plasma-membrane-associated quinones by a two-electron-transfer from initally NAD(P)H (VILLALBA *et al.* 1995). They thereby regenerate membranous hydroquinone pools depleated by radical scavenging and the redox cycling of other PMET constituents like ENOX. There are evident indications for NQO1 functions to protect cells from oxidative stress and toxic compounds, though they are also capable to produce free radicals (LI *et al.* 1995; NUTTER *et al.* 1992; SIEGEL *et al.* 2004). Additionally, it is reported to exhibit tumor-preventive and chemotherapeutic properties, as it stabilises tumor suppressors of the p53-family proteins and clearly improves the effect of certain cytotoxic drugs, particularly in breast cancer patients (ASHER *et al.* 2004; GRIM *et al.* 2012).

Another member of the PMET system associated with the inner cytoplasmatic membrane are ***cytochrome $b_5$ reductases,*** whose activity was initially observed with cytochrome b5 (STRITTMATTER 1965). It has been revealed to affect many cellular processes, ranging from the biosynthesis of fatty acid chains and cholesterol to the preservation of reduced haemoglobins in erythrocytes (HULTQUIST and PASSON 1971; KEYES and CINTI 1980; REDDY *et al.* 1977). Furthermore, the enzymes are capable of increasing intracellular concentrations of ascorbate by reducing ascorbyl free radicals (GOMEZ-DIAZ *et al.* 1997; SHIRABE *et al.* 1995). However, this one-electron transfer activity with NADH as preferred electron donors was also found to be directed towards ubiquinones in the plasma membrane (NAVARRO *et al.* 1995; VILLALBA *et al.* 1995). It is widely believed that cytochrome $b_5$ reductases decisively contribute to electron flows through the lipid

bilayers by restoring reduced forms of coenzyme Q therein, molecules with substantial roles in PMET and antioxidant preservation (SUN *et al.* 1992; VILLALBA *et al.* 1997).

***Duodenal cytochrome b*** is a further component identified more recently, though its activity has already attracted attention much earlier (ASARD *et al.* 1992; MCKIE *et al.* 2001). It is a transmembrane protein present in many mammalian cells, where it mainly participates in the metabolism of iron and the preservation of extracellular ascorbate (LATUNDE-DADA *et al.* 2002; SU *et al.* 2006). The enzymatic activity of duodenal cytochrome b is strongly dependent on ascorbate as the cytosolic electron donor and, besides reducing ferric iron for transmembrane uptake, the extracellular reduction of AFR is regarded as being the prevailing activity responsible for cellular ascorbate recycling, most strikingly in erythrocytes (SU *et al.* 2006; VANDUIJN *et al.* 2000).

The final proteins to be mentioned in this sequence are the ***voltage-dependent anion-selective channels 1 (VDAC1)***. VDACs are well known as pore-forming proteins in the outer membrane of mitochondria, mediating the flux of mitochondrial metabolites and ions like ATP, pyruvate and calcium between mitochondria and cytosol (COLOMBINI 1980; SHOSHAN-BARMATZ *et al.* 2010). In addition, they are implicated in the mitochondria-induced pathway of cell death by interacting with pro-apoptotic proteins, triggering the release of the apoptotic messenger cytochrome c from the intermembrane space (SHIMIZU *et al.* 1999). Consequently, the presence of VDACs in cellular membranes other than mitochondria asked for additional functional definitions of these proteins (GONZALEZ-GRONOW *et al.* 2003; THINNES 1992). In plasma membranes of a human cell line, VDAC1 was shown to possess NADH-ferricyanide reductase activity, and this activity was also found with mitochondrial preparations of the protein (BAKER *et al.* 2004). It is discussed that the reductive activity seen with VDAC1 might in part be the consequence of its pore-forming nature, allowing cytosolic reducing equivalents to be passively released (DEL PRINCIPE *et al.* 2011). However, as VDAC1 has protein sequences typical for catalytic domains of NADH-ferricyanide reductases, its enzymatic participation in the transplasma membrane transfer of electrons cannot be so easily dismissed (BAKER *et al.* 2004).

## 1.2 ENOX protein family

ENOX (ECTO-NADH oxidase) proteins are enzymes anchored to the outer side of plasma membranes and are thought to be one of the final oxidases in plasma membrane electron transport systems (KISHI *et al.* 1999; MORRE 1995). They mediate the transfer of initial intracellular electrons from NAD(P)H to reduce extracellular substrates with intramembranous ubiquinones ($Q_{10}$) serving as intermediate electron acceptors (figure 2). The extracellular substrates can be dissolved molecular oxygen or disulfide-bonds of proteins, which are situated either at the membrane itself or in the extracellular matrix. Additionally, ENOX proteins can perform protein disulfide-thiol interchanges by oxidating the thiol group of one protein and transferring the electrons onto disulfide-bonds of another (MORRE 1994; MORRE 1998). Both activities, the oxidation of hydroquinone ($Q_{10}H_2$) on the one hand and the protein disulfide-thiol interchange on the other hand, alternate and are unique properties of all members of the ENOX protein family, serving to distinguish them from other NADH-dependent oxidases (BRIGHTMAN *et al.* 1992; MORRE and MORRE 1995). Another characteristic feature of ENOX proteins is the oscillatory pattern observed for these activities, which are not constantly present but arise in always the same time intervals (BACON and MORRE 2001; PETER *et al.* 2000; WANG *et al.* 2001). Depending on the type of ENOX protein, these intervals range from 22 to 26 minutes.

ECTO-NADH oxidases can dissociate from the plasma membrane, probably by proteolytic cleavage, and freely circulate in all kinds of body fluids like urine, saliva and blood, though in truncated forms, without losing their activity (BERRIDGE and TAN 2000; YANTIRI *et al.* 1998). Since two members of the enzyme family (namely arNOX and tNOX) are associated with cancer and aging-related pathologies, their examination in urine and blood samples of patients may provide an easy and fairly noninvasive method indicating increased risk of disease and could essentially contribute to a more effective early diagnosis (MORRE *et al.* 2008a; MORRE and MORRE 2006a).

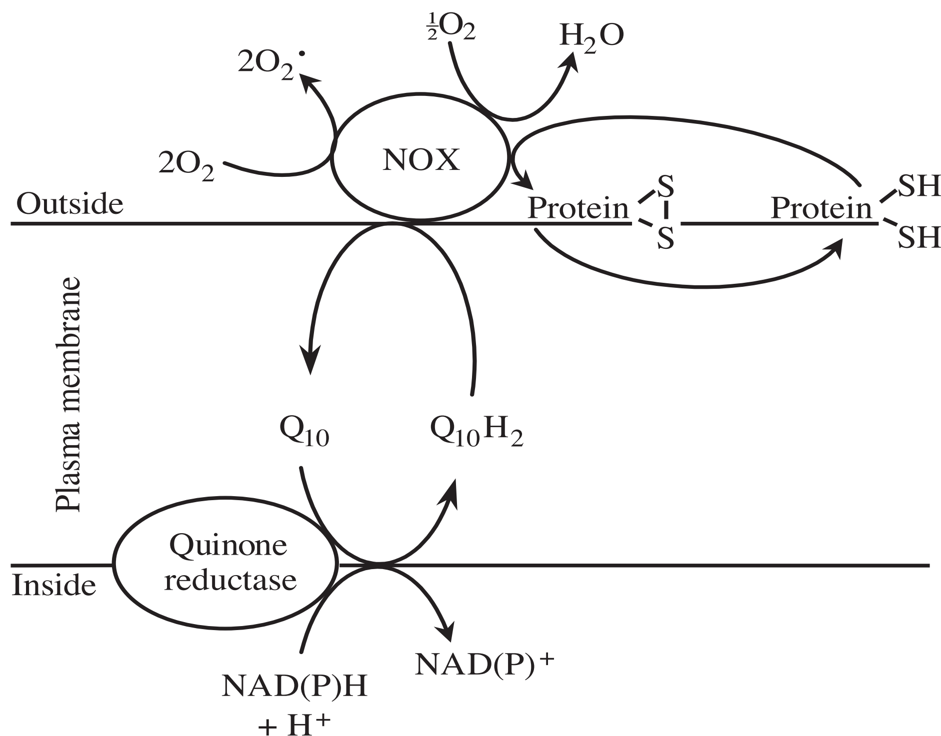


**Figure 2: The function of ENOX in transplasma membrane electron transports.** Initial electrons are transferred from cytosolic NAD(P)H to intramembrane-located ubiquinone ($Q_{10}$), probably by a quinone reductase situated at the inner side of the plasma membrane. The reduced ubiquinones ($Q_{10}H_2$) serve as electron carriers, passing their electrons on to ENOX (NOX) situated at the outer side of the plasma membrane. There, ENOX proteins use these electrons to reduce extracellular molecular oxygen to water and superoxide, respectively, and to resolve disulfide-bonds of proteins into thiol groups. Apart from that, ENOX proteins also mediate the interchange of thiol groups and disulfide-bonds between proteins. The superoxide production refers to activities observed only with arNOX. Illustration was taken from „Suface oxidases and oxidative stress propagation in aging“ (MORRE *et al.* 2000).

So far, the ENOX protein family comprises four members, denoted as ENOX1 to ENOX4. ENOX1, or CNOX as the constitutive NOX, is present in many organisms, including animals and plants. Its activity oscillates in 24-minutes-intervals and can be influenced by hormones and growth factors (BRUNO *et al.* 1992; JIANG *et al.* 2008).

ENOX2 is the tumor-associated activity (tNOX) found only in transformed cells and cells of cancers with an oscillatory pattern of 22 minutes (CHUEH *et al.* 2002). It appears to be sensitive to several drug-treatments but is not or only slightly stimulated by hormones or growth factors (BRUNO *et al.* 1992; MORRE *et al.* 1996; MORRE *et al.* 1995b).

The activity of the third member (ENOX3) is named age-related NOX or arNOX because it is nearly exclusively present in individuals beyond the age of 30 and then increases with advancing age (MORRE *et al.* 2010). In contrast to the other three protein family members, it does not reduce $O_2$ to water but preferentially to superoxide, and this activity reoccurs every 26 minutes (MORRE and MORRE 2006a; MORRE *et al.* 2003b). The particular properties of arNOX and its detection will be discussed in detail in chapter 1.2.2.

The fourth and final ENOX protein, ENOX4, is associated with plants and has an oscillatory activity pattern of 24 minutes, regulated by plant-specific growth hormones like auxine (CHUEH *et al.* 1997; MORRE *et al.* 2003a).

### 1.2.1 The functional roles of ENOX proteins

***Restoration of $NAD^+$ pools***

The ratio of NAD(P)H to $NAD(P)^+$ in the cytosols of healthy cells is strictly regulated to maintain proper cellular processes like energy metabolism, gene expression and signalling pathways (KOCH-NOLTE *et al.* 2009; RUTTER *et al.* 2001; STUBBS *et al.* 1972). Constantly altered ratios of the nicotinamide-adenine dinucleotide couple are associated with pathological conditions like diabetes and can interfere with cell death control mechanisms (ALANO *et al.* 2004; IDO 2007). Defective or absent mitochondrial respiration is commonly compensated with high glycolytic activity consuming cytosolic $NAD^+$ to provide sufficient energy, with the effect that the level of cytosolic NADH increases (LEE and WEI 2009).

Many indices suggest that ENOX proteins are key enzymes for the regeneration of intracellular $NAD^+$ when the function of mitochondria, particularly during aging, is impaired (LARM *et al.* 1994; LAWEN *et al.* 1994; TRIFUNOVIC and LARSSON 2008).

***Regulation of cell enlargement***

ECTO-NADH oxidases seem to have a critical influence on the enlargement phase of cells (MORRE 1998; MORRE and MORRE 2006b). Before mitosis, each cell has to double the amount of diverse organelles and as a consequence increases in size in order to end up in two fully functional cells after cell division (MITCHISON 2003).

It was shown that the stimulation of ENOX activity by growth factors and hormones linearly correlated with enhanced cell enlargement and subsequent division of cells. Moreover, the enlargement phase was not a continuous process but happened stepwise in time intervals corresponding to the oscillatory activity pattern of the enzyme (MORRE *et al.* 2001; POGUE *et al.* 2000). This evident correlationship was observed for the constitutive ENOX (CNOX) and the auxin-associated ENOX in plants (MORRE *et al.* 2001; WANG *et al.* 2001).

In transgenic mice, the overexpression of tNOX significantly enhanced growth and enlargement not only of cells but of the whole organism (YAGIZ *et al.* 2008). When the activity of ENOX was dramatically reduced by known inhibitors, cells were unable to gain size, stopped to divide and sometimes underwent apoptosis (GRIECO and MORRE 1999; MORRE *et al.* 1995a). In this context, especially tNOX might serve as a promising molecular target for anticancer therapies to inhibit tumor cell proliferation (DAVIES and BOZZO 2006). By now, there are no evidences for an influence of the age-related ENOX (arNOX) on cell growth and enlargement.

***Ultradian clocks of the circadian rhythm***

All living organisms are dominated by a biological clock controlling periodically recurring as well as linearly proceeding events within a lifetime. This includes behavioural and motility patterns, embryonic development, ovulation and many other cellular and intracellular processes like heart beat, hormone release, cell cycle, metabolism, etc. (RENSING *et al.* 2001; SCHIBLER 2006). The changes of external physical parameters like light, temperature and lunar gravity create the basic conditions for the timing of daily, monthly and seasonal occurring biological phenomenons, but are insufficient to solely coordinate processes with higher periodicity like rhythmic mussle contractions or the

cyclic activity of many enzymes. An inherent timekeeping system within the circadian rhythm of 24 hours, the ultradian clock, is suggested to regulate the exact timing and repeats of diverse biological processes up to milliseconds by serving as an oscillating pulser (BELL-PEDERSEN *et al.* 2005; LLOYD and KIPPERT 1993).

ECTO-NADH oxidases possess an exactly timed oscillating activity, which was shown to at least influence growth behaviour of cells (MORRE *et al.* 2001; POGUE *et al.* 2000). In particular ENOX1 and ENOX4, the constitutive and auxin-induced forms of ENOX, respectively, are suspected of being such timing pulsers (MORRE *et al.* 2002a). Their activity oscillates in 24 min periods, which, when multiplied with 60, defines a day length, and studies in yeast and plants support this hypothesis (DICK *et al.* 2013; MORRE *et al.* 2002b).

### 1.2.2 Age-related NADH oxidases

The identification of an age-related NADH-dependent hydroquinone oxidase (arNOX) dates back one decade, when Morré and colleagues found a cell surface oxidase, whose activity pattern oscillated just as characteristic for members of the ENOX protein family, but was only present in cells and tissues of aged individuals (MORRE *et al.* 2003b; MORRE and MORRE 2003b). Moreover, this oxidase was reducing extracellular $O_2$ to superoxide in oscillating time periods of 26 minutes, properties, which markedly differed from the yet described ECTO-NADH oxidases and emphasised the existence of a new ENOX member.

The activity of superoxide production was measured by a standard method using ferricytochrome c and superoxide dismutase (SOD) (BUTLER *et al.* 1982). The oxygen radical reduces ferricytochrome c to ferrocytochrome c, which is measured spectrophotometrically. Since ferricytochrome reduction can also occur independently of superoxide, SOD is added. Thereby, arNOX activity was determined as the rate of ferricytochrome c reduction inhibited by SOD and defines its activity ever since.

The presence of arNOX was further analysed in serum, buffy coats, skin and saliva of human subjects, always with the same result. In young subjects (up to 30 years), the enzyme's activity was not or hardly detectable, whereas aged subjects (40 years and older) had clearly detectable activities, which significantly increased with age (MORRE *et al.* 2008b; MORRE *et al.* 2010). The evident correlation of arNOX appearance and activity level with advancing age and the consequent increase in extracellular superoxide production is suggested to be related to aging-induced cellular damage and diseases (MORRE and MORRE 2003a; MORRE and MORRE 2006a).

Aging is a process defined by a continuous decline of cellular function and proliferation. It is thought to be triggered by the accumulation of reactive oxygen species, which are accidently but steadily produced by the respiratory chain in mitochondria and generated by diverse harmful pollutants encountered in daily life (OLIVEIRA *et al.* 2010). Furthermore, mitochondrial defects occur more often with aging and can contribute to elevated levels of superoxide (TRIFUNOVIC and LARSSON 2008). Though organisms have evolved several mechanisms to remove these reactive molecules, the oxidative damages on cells and proteins are evident (MIURA and ENDO 2010). Lipid peroxidations for example increase with age and can cause pathologic thickenings of arteries, as seen with atherosclerosis (ESTERBAUER *et al.* 1993). Also the emergence of many neurodegenerative diseases and cancers in advancing age is associated with accumulating oxidative stress (CUTLER 1992; EMERIT *et al.* 2004).

Coenzyme $Q_{10}$, an essential cellular antioxidant, can inhibit the activity of arNOX and was shown to decelerate oxidative damages and improve the elasticity of aged skin (KERN *et al.* 2010; MORRE *et al.* 2008c). It is already commercially used as an aging-protective agent in the food and cosmetic industry (JEYA *et al.* 2010; KERN *et al.* 2010).

#### ***1.2.2.1 arNOX and the TM9 superfamily of transmembrane proteins***

Transmembrane 9 superfamily proteins (TM9SF) are distributed in tissues of many species, including humans, plants and yeast. They consist of a large hydrophobic N-terminal sequence and a highly conserved C-terminus containing nine transmembrane

domains (CHLUBA-DE TAPIA *et al.* 1997). Their functions are yet described rather vague, but it is suggested that they are integral membrane proteins of endocytic compartments and to be involved in the innate immune response (PRUVOT *et al.* 2010; SCHIMMOLLER *et al.* 1998; SINGER-KRUGER *et al.* 1993). To date, five subtypes of TM9SF are reported to exist in humans (CHLUBA-DE TAPIA *et al.* 1997; SCHIMMOLLER *et al.* 1998; TANG *et al.* 2013). Among the ENOX protein family, arNOX is the only known to also possess a nine-transmembrane-domain motif at its C-terminus, and, though it is a strongly conserved motif, this was the first hint to TM9SF proteins (MORRE and MORRE 2013).

The idea to seriously associate arNOX activity with TM9SF proteins came up, when Tang and colleagues sequenced N-terminal peptides of the isolated oxidase and database search revealed one corresponding protein, namely TM9SF4 (TANG *et al.* 2013). They could show that recombinantly expressed human TM9SF isoforms exhibited the same specific activity with the same oscillating pattern as characteristic for arNOX. Subsequent immunoblottings with arNOX-positive sera and saliva were confirming the presence of the five yet known human isoforms of TM9SF. Additionally, each of these isoforms could be correlated to one of the five distinct activity maxima constantly observed with arNOX (MORRE *et al.* 2010). The study of Tang et al. thereby demonstrated that in humans, the five isoforms of arNOX activity originate from the five isoforms of the TM9 superfamily of transmembrane proteins.

As TM9SF proteins were reported to localise to endosomes, this would also explain why the activity of arNOX is absent in young subjects (SINGER-KRUGER *et al.* 1993). It is possible that certain processes associated with aging might trigger the re-localisation of arNOX at the outer face of plasma membranes, probably by exocytosis, but this is still hypothetical.

## 1.3 Aim of the project

The work in hand aimed to add to the current knowledge about transplasma membrane electron redox systems in human erythrocytes by pursuing three basic approaches. First, we tried to identify arNOX as an additional component of the PMET system in red blood cells, particular with aged subjects. Morré and colleagues have already reported the existence of this oxidase in buffy coats and sera of aged individuals, so we applied spectrophotometrical measurements according to the method published for arNOX activity detection (MORRE *et al.* 2003b; TANG *et al.* 2013). In addition, membrane preparations of aged blood donors were immunologically analysed to detect plasma-membrane-associated forms of arNOX.

The second attempt intended to compare transmembranous electron transfer activities in erythrocytes of several male and female donors to find out whether differences in reductive capacities can be linked to a gender-dependent context.

Finally, we analysed PMET activities in relation to extracellularly changing conditions most probably inducing an intracellular response. For this purpose, we tested, e.g., the effect of increased osmolaric stress and lipid alterations in the plasma membrane as well as antioxidant and excess glucose supplementation. Furthermore, the impact of prolonged blood storage on this particular redox system was analysed. To assay the corresponding reductive activities, two different potential electron acceptors were added, ferricyanide and ferricytochrome c. This aimed to detect possible preferences for the reduction of one or the other compound in general and in consequence of the just mentioned treatment procedures, which might indicate the existence of different PMET systems and/or mechanisms. Ferricytochrome c was never employed before for this purpose, so its use was particularly established for our measurements with red blood cells.

# 2 Material and Methods

## 2.1 Buffers and solutions

**PBS (phosphate buffered saline)**

- 150 mM NaCl
- 10 mM phosphate buffer
- adjusted to pH = 7.4 with HCl

**PBSG**

- PBS with 7.5 mM glucose

**TBS (tris buffered saline)**

- 150 mM NaCl
- 10 mM Tris buffer
- adjusted to pH = 7.4 with HCl

**TBSG**

- TBS with 7.5 mM glucose

**5x Laemmli sample buffer**

- 0.3 M Tris base
- 10 % SDS (w/v)
- 50 % glycerol
- bromphenole blue until dark blue
- adjusted to pH = 6.8 with HCl
- 10 % ß-mercaptoethanol,
  added before use

**SDS running buffer**

- 25 mM Tris base
- 190 mM glycine
- 0.1 % SDS
- adjusted to pH = 8.3 with HCl

**Transfer buffer**

- 25 mM Tris base
- 190 mM glycine
- 20 % methanol

**TBST**

- TBS with 0.05 % (v/v) Tween 20

**Blocking solution (in TBST)**

- 1 % PVP (w/v)
- 1 % skimmed milk powder (w/v)

## ***Hank's Buffered Salt Solutions (HBSS)***

**Stock #1**

- 100 ml $H_2O$
- 8.0 g NaCl
- 0.4 g KCl

**Stock #2**

- 100 ml $H_2O$
- 0.358 g $Na_2HPO_4$
- 0.60 g $KH_2PO_4$

**Hank's Buffered Salt Solution (HBSS) Premix - solutions were combined in the following order:**

- 10 ml Solution #1
- 1 ml Solution #2
- 1 ml Solution #3
- 86 ml distilled $H_2O$
- 1 ml Solution #4

**Hank's Buffered Salt Solution (HBSS) full strength (mixed prior to use)**

- 9.9 ml Hank's Premix
- 0.1 ml Stock #5

**Stock #3**

- 50 ml $H_2O$
- 0.72 g $CaCl_2$

**Stock #4**

- 50 ml $H_2O$
- 1.23 g $MgSO_4$ x $7H_2O$

**Stock #5**

- 10 ml $H_2O$
- 0.35 g $NaHCO_3$

**HBSS-Glc**

- HBSS full strength with 5 mM glucose

**DPBSE**

- Dulbecco's Phosphate-Buffered Saline with 5 mM EDTA

***Developing solution***

**3 M sodium acetate, pH = 6.5**

- 4.082 g sodium acetate trihydrate
- ad 10 ml with $ddH_2O$ after adjusting pH to 6.5 with glacial acid
- stored at room temperature

**0.2 M citrate**

- 192 mg citrate
- ad 5 ml with $ddH_2O$
- stored at room temperature

**3.3 mM $FeCl_3$**

- 1.784 mg $FeCl_3$ hexahydrate
- ad 2 ml with $ddH_2O$
- stored at 4°C for one week

**6 mM bathophenanthroline**

- 33.3 mg bathophenanthroline
- ad 10 ml with $ddH_2O$
- stored at 4°C for one to two weeks

**Table 1: Preparation of 10 % SDS-PAGE gels** (amounts stated for four gels in ml):

| reagents | stacking gel | seperating gel |
|---|---|---|
| $ddH_2O$ | 4.1 | 7.9 |
| 30 % Acrylamid-Bis-acrylamid mix | 1.0 | 6.7 |
| 1.5 M Tris buffer, pH = 8.8 | - | 5.0 |
| 1 M Tris buffer, pH = 6.6 | 0.75 | - |
| 10 % SDS | 0.6 | 0.2 |
| 10 % APS | 0.6 | 0.2 |
| TEMED | 0.006 | 0.008 |

**Table 2: Specific substances* used for assays and treatment procedures**

| systematic name | product number | common name/abbreviation |
|---|---|---|
| *Potassium hexacyanidoferrat(III)* | P3667 | ferricyanide |
| *Potassium hexacyanidoferrat(II) trihydrate* | P9387 | ferrocyanide |
| *Bathophenanthrolinedisulfonic acid disodium salt hydrate* | B1375 | bathophenanthroline |
| *Cytochrome c (oxidised form)* | C2037 | cytochrome c |
| *Superoxide dismutase* | S7446 | SOD |
| *Methyl-ß-cyclodextrin* | C4555 | cyclodextrin |
| *Sodium orthovanadate* | S6508 | vanadate |
| *Coenzyme $Q_{10}$* | C9538 | $CoQ_{10}$ |

** all purchased from Sigma-Aldrich®*

## 2.2 Blood donations and storage

Blood was obtained either from voluntary donors or provided by the Austrian Red Cross, both collected in EDTA-coated vacuum tubes. Unless used immedeately, the blood was stored at 4°C. Blood samples from the Austrian Red Cross varied in age between 2 and 6 days post donation, whereas donors termed „young“ were less than 30 years old and donors termed „aged“ were older than 50 years.

## 2.3 arNOX activity and protein analysis

### 2.3.1 Red blood cell isolation

Freshly collected blood from one „young“ and „aged“ donor (4 ml each) was centrifuged for 20 min at 200 x g. The supernatant (plasma) and buffy coat were removed generously. 2 x 200 µl of both - „young“ and „aged“ - erythrocyte suspensions were washed twice in 1 ml DPBSE each and centrifuged at 16,000 x g for 20 sec.

The clear supernatant was removed and the erythrocyte suspensions were pooled according to their origin, „young“ or „aged“.

### 2.3.2 Lysis and membrane preparation

100 µl of washed erythrocyte pellet was mixed with 1 ml icecold 5 mM phosphate buffer (pH = 8), incubated for 5 min and centrifuged for 10 min at 16,000 x g. The liquid above the cloudy pellet-border (slightly visible when holding against light) was removed, the membrane pellet (= ghosts) was washed four times with 1 ml icecold 5 mM phosphate buffer, each washing step followed by 10 min centrifugation at 16,000 x g.

To remove cytoskeletal proteins, which were still associated with plasma membrane preparations, 100 µl of washed ghosts were suspended in 800 µl 0.1 M NaOH and incubated for 15 min on ice. Then, samples were centrifuged for 10 min at 16,000 x g and ghost pellets washed once with icecold 5 mM phosphate buffer as described in the previous paragraph. All steps were performed at 4° C.

### 2.3.3 Western blot analysis

Erythrocyte membranes (ghosts) of „young“ and „aged“ donors were suspended in 5 x Lämmli sample buffer, incubated at 95°C for 5 min and loaded onto a 10 % SDS-PAGE gel (5 µl and 10 µl for each sample) together with 3 µl of marker proteins (PageRuler Prestained, Thermo Scientific). Running time was 45 min at 200 V.

Afterwards, the proteins on the gels were transfered onto nitrocellulose membranes (Whatman Protran BA 83 Nitrocellulose Membranes) for two hours at 4°C and 80 V. The membranes were then put into a blocking solution for one hour at room temperature (RT) with slight shaking. The blocked membranes were washed once for 1 min and four times for 5 min with TBST. 7 ml of first antibody (Ab) solution (polyclonal Rb-anti-TM9SF1, TM9SF2 and TM9SF3, purchased from the laboratory of James Morré, 1 : 1,000 dilution in TBST + 0.05 % $NaN_3$) were added to each membrane in 50 ml falcon tubes, incubation was performed over night at 4°C with slight shaking. The membrane for negative control was incubated without antibodies.

Membrane washing: 1 x for 1 min and 4 x for 5 min with TBST.

The second antibody (HRP-conjugated Pierce Goat anti-Rabbit IgG, Thermo Scientific, 1 : 10,000 dilution in TBST) was added in the same volume like the first Ab and incubated for one hour at RT with slight shaking.

Membrane washing: 1 x for 1 min and 3 x for 5 min with TBST, final washing was with TBS for 1 min. Membranes were incubated with substrate (ECL plus Western, GE Healthcare) for three to four minutes and put onto a radiosensitive film, which was then developed in a developing machine.

### 2.3.4 arNOX activity measurement

Whole blood of aged and young subjects was given by the Austrian Red Cross. To isolate red blood cells (RBC), blood was centrifuged (20 sec at full speed), the plasma and buffy coat were removed generously. The RBC pellet was then washed three times with PBS and resuspended 1 : 10 in PBSG.

The assay for determining arNOX activity is a modified version of the method described by Morré et al. (MORRE *et al.* 2003b) and was performed as follows: 200 µl of the RBC-suspension was mixed with 196 µl of 73.5 µM cytochrome c solution (in PBSG) and in parallel either 4 µl of dd$H_2O$ or SOD (2 U/µl in dd$H_2O$).In summary, all samples (exclusive those used for control) contained 20 µl RBC-pellet, 36 µM cytochrome c and 8 U SOD.
Samples were incubated for 30 min at RT with mild agitation. At timepoints 0 min and 30 min, 200 µl aliquotes were taken, shortly spun down and 160 µl supernatant was taken off for absorbance measurement in the dual wavelenght mode at 550 nm and
540 nm (spectrophotometer Hitachi U-3501). Two control samples, lacking either RBCs or cytochrome c, were included for all measurements.
To calculate the respective cytochrome c reduction activities, absorbance values obtained at 540 nm were subtracted from those at 550 nm and the difference between 0 min and 30 min of incubation was calculated. Finally, values of control samples were subtracted and the subsequent absorbance values normalised to the amount of cells and the time of incubation applied for aliquotes. Thereby, the cytochrome c reduction activity is expressed as $(A_{550} - A_{540})/min^{x}\ ml\ RBC$ (for a detailed scheme of calculation see table 4 in chapter 3.1).
For determining the proportion of SOD-sensitive activity, values obtained from samples with SOD were subtracted from those without SOD.

## 2.4 Analysing the plasma membrane redox system in erythrocytes

### 2.4.1 Preparation of red blood cells

Blood was collected from voluntary donors. Whole blood was centrifuged for 20 sec at full speed, the supernatant and buffy coat were taken off generously.
The RBC pellet was washed three times with TBS and resuspended 1 : 10 in TBSG for immediate use in subsequent assays unless specified otherwise.

### 2.4.2 Measuring electron transport across the red blood cell plasma membrane

To determine the plasma membrane electron transport activity in erythrocytes, we applied two different methods, which basically differ in the kind of electron acceptor used. Ferricyanide, the first electron acceptor, was prepared in a 50 mM stock solution (dissolved in $ddH_2O$) and stored at 4°C for about two weeks, protected from light. The second electron acceptor used was the fully oxidised form of cytochrome c, also called ferricytochrome c, which was prepared in a 6 mM stock solution (dissolved in $ddH_2O$) and stored at 4°C for about one week.

#### *2.4.2.1 Ferricyanide reduction assay*

The method to analyse ferricyanide reduction rates was first described by Avron et al. (AVRON and SHAVIT 1963). We modified the protocol for the spectrophotometrical measurement and established $Fe^{2+}$-complex-formation kinetics to determine the amount of ferrocyanide produced. This was due to preliminary experiments with plasma membrane ghosts, which required a fast detection of ferrocyanide present after a defined incubation period. The preincubation with bathophenanthroline prior to measurements would have been incompatible with this aim, since ghosts were treated with Triton X-100 and thus were not seperable anymore from the samples. However, as already successfully established, this method was retained also in assays involving intact cells.
100 µl of RBC suspension (see 2.4.1) was added to 100 µl 0.7 mM ferricyanide solution (in TBSG) and incubated for 30 min at RT and mild agitation. The final concentration of ferricyanide for each sample was always 350 µM. Two control samples, lacking either RBCs or cytochrome c, were included in parallel. At timepoints 0 min and 30 min of incubation, 100 µl aliquotes were taken and shortly spun down to remove RBCs. 75 µl of the supernatant were pipetted into a quarz cuvette and mixed with 75 µl developing solution (1 : 1.5 : 1.5 : 3 : 3 mixture of $ddH_2O$, 3.3 mM $FeCl_3$, 0.2 M citrate, 3 M sodium

acetate and 6 mM bathophenanthroline, freshly prepared before use). Immediately after mixing, the increase in absorbance at 535 nm was recorded for 60 sec (spectrophotometer Hitachi U-3501).

The slope of the resulting curve between 50 sec and 60 sec was used to calculate the amount of ferrocyanide produced with the help of ferrocyanide standard solutions. To determine the actual ferricyanide reduction activity in erythrocytes, the difference between 0 min and 30 min of incubation was calculated and controls were subtracted. The activity was normalised to the amount of cells present in aliquotes and the time of incubation applied and was expressed as *nmol ferrocyanide/min $^x$ µl RBC* (for a detailed scheme of calculation see table 3).

**Table 3: Calculation of ferricyanide reduction activity.**
**(**For the purpose of illustration an example of one measurement is presented.**)**

| sample | time (min) | $A_{535}$ (50 sec) | $A_{535}$ (60 sec) | 60 - 50 sec | 30 - 0 min | - (-ferri-cyanide) | - (-RBC) | µmol ferro-cyanide* |
|---|---|---|---|---|---|---|---|---|
| RBC | 0 | 0.383 | 0,388 | 0.005 | 0.051 | 0.049 | 0.048 | 0.008 |
| | 30 | 1.113 | 1.169 | 0.056 | | | | |
| -ferricyanide | 0 | 0.374 | 0.377 | 0.003 | 0.002 | | | |
| | 30 | 0.420 | 0.425 | 0.005 | | | | |
| - RBC | 0 | 0.316 | 0.317 | 0.001 | 0.001 | | | |
| | 30 | 0.364 | 0.366 | 0.002 | | | | |
| activity** (nmol ferrocyanide/min $^x$ µl RBC) | | | | | | | 0.053 | |

* *if the linear equation of the ferrocyanide standard curve was y = 6 $^x$ x µmol ferrocyanide.*

** *calculated for a 30-minute-assay and 5 µl RBC present in the aliquote taken for the measurement.*

#### *2.4.2.2 Cytochrome c reduction assay*

This assay is a modified version of the method described by Morré et al. (MORRE *et al.* 2003b). 200 µl of RBC suspension (see 2.4.1) was added to 200 µl 72 µM cytochrome c solution (in TBSG) and incubated for 30 min at RT and mild agitation. Two control samples, lacking either RBCs or cytochrome c, were included in parallel. At timepoints 0 min and 30 min of incubation, 200 µl aliquotes were taken and shortly spun down to remove RBCs. 160 µl of the supernatant were pipetted into a quarz cuvette and absorbance at 540 nm and 550 nm (dual wavelength mode, spectrophotometer Hitachi U-3501) was recorded.
Cytochrome c reduction activity was expressed as $(A_{550} - A_{540})/min \times ml\ RBC$ (for a detailed scheme of calculation see table 4 in chapter 3.1).

### 2.4.3 Preparation and activity analysis of haemolysates

RBC pellet was diluted 1 : 20 (to imitate the final dilution of RBCs in the assay, which was also 1 : 20) with icecold 5 mM Tris buffer (pH = 7.4), left on ice for 6 min and centrifuged for 10 min at 16,000 rpm, 4°C. The supernatant ( = 100 % haemolysate, assuming that all cells have burst) was transferred into a new eppendorf tube and diluted in TBSG to yield 2 % and 4 % haemolysate.
Both haemolysate dilutions were used for cytochrome c reduction measurement alike RBCs (see chapter 2.4.2.2), which were analysed at the same time.
Note: For starting the assay, the haemolysates were diluted 1 : 1 with cytochrome c plus buffer as described in chapter 2.4.2.2, so the final percentage of haemolysis to be imitated was 2 % and 1 %, respectively!

### 2.4.4 Pretreatment procedures of red blood cells

RBCs were prepared as described in 2.4.1. During incubation with the respective compound, the concentration of RBC was always 10 % (pelleted cells) in TBSG unless specified otherwise. After incubation, RBCs were washed twice with TBS (or PBS) and the pellet was diluted 1 : 10 with TBSG (or PBSG) for subsequent reduction assays (see chapter 2.4.2 for details).

***Cyclodextrin***

RBCs at a haematocrit of 5 % were incubated with cyclodextrin in final concentrations of 0.25 % and 0.5 % (w/v), incubation was for 20 min at RT on a slowly inverting rotating machine. Control samples contained TBSG only.
Blood of one female and one male donor was analysed for ferricyanide and cytochrome c reduction activity, respectively, within a maximum of 48 hours post donation (see chapter 2.4.2).

***Vanadate***

Blood was given by the Austrian Red Cross, from two to six day post donation.
RBCs were incubated with HBSS-Glc containing 50 µM vanadate for 30 min at 37°C on a slowly inverting rotating machine. Instead of diamide, dd$H_2O$ was added to control samples. After incubation, cells were washed twice with PBS and resuspended in PBSG. Cell number was determined in a neubauer counting chamber. 3 x $10^7$ cells were used per sample for ferricyanide reduction measurement (see chapter 2.4.2), the assay buffer was PBSG. Activity was calculated per $10^7$ cells.

***Glucose***

RBCs were incubated in TBSG containing 2 mM and 12 mM glucose for 30 min at 37°C with mild agitation. Control samples without RBCs were included.
Assays for cytochrome c and ferricyanide reduction (see chapter 2.4.2) were performed directly following this preincubation, without additional washing steps. Blood of one male and one female donor were analysed within 28 hours post donation.

***Hyperosmolarity***

Erythrocytes were suspended in two different TBSG buffers. The first buffer was conventional TBSG for control whereas in the second one, the concentration of saline was doubled to yield 300 mM NaCl for hypertonic conditions. Incubation was performed for 40 min at 37°C with slow inversions on a rotating machine. Cells were washed once with TBS and resuspended again in the same buffer used for preincubation.
The activity of both, ferricyanide and cytochrome c reduction, was analysed as described in 2.4.2, with the exception that the iso- and hypertonic conditions, respectively, were maintained during the whole assay.
RBCs of one male and one female donor were analysed within 6 hours post donation.

***Coenzyme $Q_{10}$***

Whole blood of a male and a female donor (within 48 hours post donation) was centrifuged for 15 min at 3000 rpm and 4°C. The plasma was pipetted into a new eppendorf tube, the erythrocyte pellet was stored at 4°C for later use after generously removing the buffy coat. The plasma was preincubated with 0.4 µM, 4 µM and 40 µM $CoQ_{10}$ for 30 min at 37°C and light shaking. In detail, 99 µl of plasma was mixed with 1 µl of 40 µM , 400 µM and 4 mM $CoQ_{10}$ stock solutions (dissolved in acetone). Control samples obtained acetone only.
Meanwhile, the erythrocyte pellet was washed three times with TBS, then suspended

1 : 1 in $CoQ_{10}$-preincubated plasma and incubated another 30 min at 37°C with mild agitations. Afterwards, RBCs were washed again twice and resuspended in TBSG at a haematocrit of 10 % for subsequent cytochrome c reduction assay (see chapter 2.4.2.2).

### 2.4.5 Statistical analysis

Statistical evaluations were performed using the software *„IBM SPSS Statistics 19“.* An *unpaired Student's t-test for the equality of means* was employed to analyse significant differences in the reductive activities obtained with male and female donors. The analysis of correlations between specific activities was performed with the same software.

# 3 Results

## 3.1 Establishment of a workable method for the reliable determination of ferricytochrome c reduction activity in erythrocytes

Working with red blood cells naturally involved that we always had to deal with small, but constantly present haemolysis, and the haemoglobin released thereby strongly absorbed at 540 nm, the wavelength applied during cytochrome c reduction measurements. This circumstance partially diminished reduction rates, which are indicated by an increase in absorbance at 550 nm relative to 540 nm (compare values before and after the correction with samples lacking cytochrome c in table 4).

An important process, hence, was the inclusion of control samples, an approach, which is quite unusual when assaying plasma membrane redox activities (see chapter 2.4.2.2). In detail, cytochrome c reduction activity was calculated by subtracting the absorbance values obtained at 540 nm from that at 550 nm, then, the difference between 0 min and 30 min of incubation was calculated. Finally, values of control samples were substracted and the subsequent absorbance values normalised to the amount of cells used and the time of incubation applied. Thereby, the cytochrome c reduction activity is expressed as $(A_{550} - A_{540})/min \times ml\ RBC$ (table 4).

This method allowed us to more precisely define reductive activities by excluding effects caused by haemoglobin but also by RBC-independent cytochrome c reduction, as the latter, interestingly, often accounted for around 10 % of total activity measured.

A further improvement was achieved in the final phase of the whole study and was applied during blood storage assays and gender-specific analyses.

It was motivated by the suspicion that haemolysed cells also release cytosolic reduction equivalents, which could reduce the added oxidants independent of plasma membrane electron transport events.

**Table 4: Calculation of cytochrome c reduction activity** (For the purpose of illustration an example of one measurement is presented)**.**

| sample | time (min) | $A_{550}$ | $A_{540}$ | $A_{550}$ - $A_{540}$ | 30 - 0 min | - (-cytochrome c) | - (-RBC) |
|---|---|---|---|---|---|---|---|
| RBC | 0 | 0.318 | 0.377 | -0.059 | 0.020 | 0.032 | 0.030 |
| | 30 | 0.366 | 0.405 | -0.039 | | | |
| -cytochrome c | 0 | 0.109 | 0.125 | -0.016 | -0.012 | | |
| | 30 | 0.159 | 0.187 | -0.028 | | | |
| - RBC | 0 | 0.220 | 0.263 | -0.043 | 0.002 | | |
| | 30 | 0.248 | 0.289 | -0.041 | | | |
| activity* (($A_{550}$ - $A_{540}$)/min $^{x}$ ml RBC) | | | | | | 0.1 | |

** calculated for a 30-minute-assay and 0.01 ml RBC present in the aliquote taken for measurement.*

To exclude the interference of haemolysis with cytochrome c activity measurements, we employed haemolysate standards, which were routinely analysed in concert with RBC samples of one and the same donor (see chapter 2.4.3).

For determining the percentage of haemolysis present during the assay with RBCs, a standard curve was generated in which the percentage of haemolysate used was plotted against the corresponding absorption obtained at 540 nm with controls lacking cytochrome c (figure 3A). Haemoglobin has an absorption maximum near 540 nm, therefore, the absorption at 540 nm is a good indicator for the extent of occurring haemolysis. Since haemolysis of RBCs increased with increasing time of incubation, the absorptions read at 540 nm at the beginning and at the end of the assay were averaged to yield mean values for haemolysis. A second standard curve served to correlate haemolysis with cytochrome c reduction activity by plotting the percentage of

haemolysate used against the corresponding activity obtained (figure 3B). Thereby, the percentage of haemolysis in cytochrome c reduction assays could be determined and the activity resulting thereof was subtracted to yield cytochrome c reduction activities for intact RBCs only. To demonstrate the reductive capacity of haemolysates themselves, activities were calculated per percent haemolysis present (instead of per ml RBC).

Since only 10 % of all samples measured marginally exceeded 1 % of haemolysis, the reduction of RBC volumes during assays was negligible. For a detailed scheme of the calculation approach see table 5.

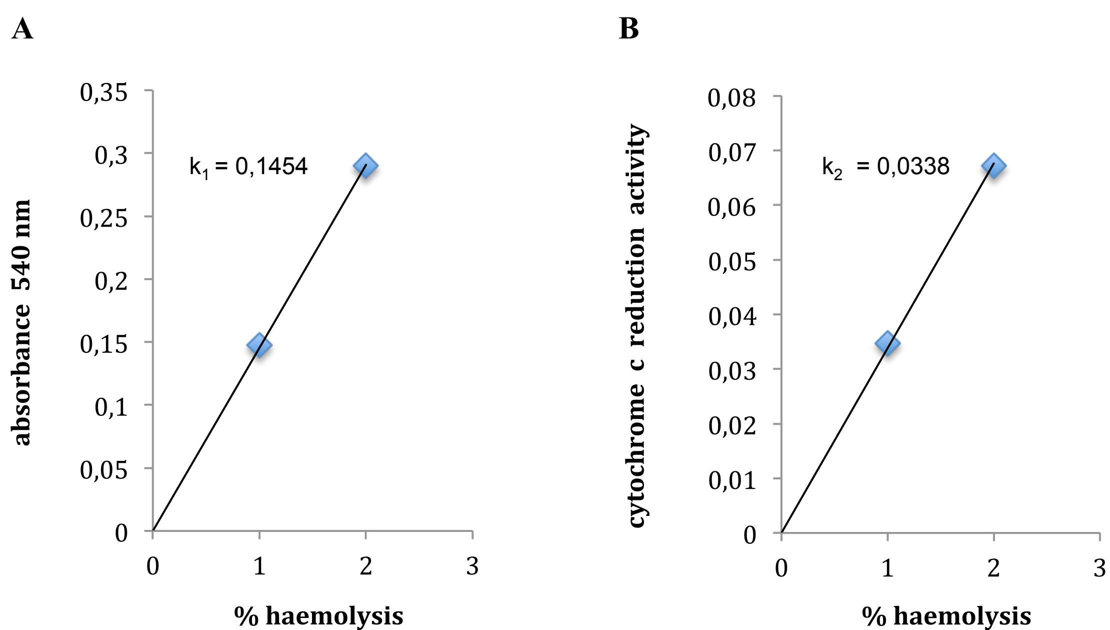


**Figure 3: Standards of haemolysates analysed for cytochrome c reduction activity.** For the purpose of demonstration, results of one donor are shown. **(A)** Absorbance at 540 nm, measured with controls lacking cytochrome c at the beginning (timepoint 0 min) and the end (timepoint 30 min) of the assay, was averaged and plotted against the percentage of haemolysis used. The resulting standard curve cuts the axes at zero, whereby $k_1$ indicates the slope of the curve. **(B)** The rate of cytochrome c reduction was plotted against the percentage of haemolysis used. The resulting standard curve cuts the axes at zero, whereby $k_2$ indicates the slope of the curve. Activity is expressed as *$(A_{550}$ - $A_{540})$/min. $^x$ ml RBC.*

Regrettably, we were not able to generate a similar correction method for ferricyanide reduction measurements, since there, the obtained activities did not correlate at all with the amount of haemolysis present in standard samples (data not shown).

**Table 5: Correction of cytochrome c reduction activity for haemolysis.** Based on haemolysate standard measurements, the results for cytochrome c reduction analyses performed with RBCs were corrected to yield haemolysis-independent activities (= final cytochrome c reduction activity).

| total activity ( $(A_{550} - A_{540})$/ min $^x$ ml RBC ) | % haemolysis | activity of haemolysate | final cytochrome c reduction activity |
|---|---|---|---|
| see table 4 for calculation | $\frac{\text{mean of } (A_{540}\,0\text{ min}, A_{540}\,30\text{ min})^*}{k_1}$ | $k_2$ $^x$ % haemolysis | total activity - activity of haemolysate |

*$k_1$ = slope of the standard curve „$A_{540}$* versus *% haemolysis“ (see figure 5A)*

*$k_2$ = slope of the standard curve „cytochrome c reduction activity* versus *% haemolysis“ (see figure 5B)*

** measured with control samples lacking cytochrome c*

## 3.2 Determinations on the possible existence of arNOX in human red blood cells

Recently, Morré and colleagues suggested that the arNOX activity originates from proteins of the TM9 superfamily (in humans designated TM9SF1 to TM9SF5), since they found out that TM9SF proteins were NADH-dependent hydroquinone oxidases with specific oscillatory activity patterns, as already known with arNOX (see chapter 1.2.2.1). Further investigations revealed that the five yet characterised isoforms of arNOX are

identical with the five human isoforms of TM9SF (TANG *et al.* 2013).

By now, arNOX activity was never described to exist in human red blood cells. Therefore, membranes of RBCs were examined for the presence of arNOX by immunolabelling them with antibodies raised against three members of TM9SF, which were TM9SF1, TM9SF2 and TM9SF3. These antibodies were generated to recognise specific extracellular regions of the respective TM9SF protein and were purchased from James Morré himself. Additionally, we analysed RBCs for the proportion of SOD-inhibitable cytochrome c reduction activity, which, according to Morré et al., is the standard method to measure arNOX activity (see chapter 2.3.4).

For comparison, blood samples were always from young (< 30 years) and aged (> 50 years) subjects.

### 3.2.1 arNOX protein analysis

Plasma membranes of RBCs were prepared and analysed as described in chapters 2.3.2 and 2.3.3. The western blot analysis revealed that any of the TM9SF antibodies used was binding highly unspecific to the same two erythrocyte membrane proteins, which could be the anion transporter (at around 90 kDa) and actin (at around 45 kDa) as well as in part to a 55 kDa membrane protein (figure 4). The expected signals at 67 kDa for TM9SF1 and TM9SF3 and 76 kDa for TM9SF2 are totally missing.

Erythrocytes have lots of cytoskeletal proteins anchored to its plasma membrane, which could interfere with the detection of less abundant proteins.

To enrich the proportion of transmembrane proteins (as TM9SF proteins are suchlike), we repeated this experiment with membranes having the vast majority of cytoskeletal proteins removed. Regrettably, this procedure did not improve results (data not shown).

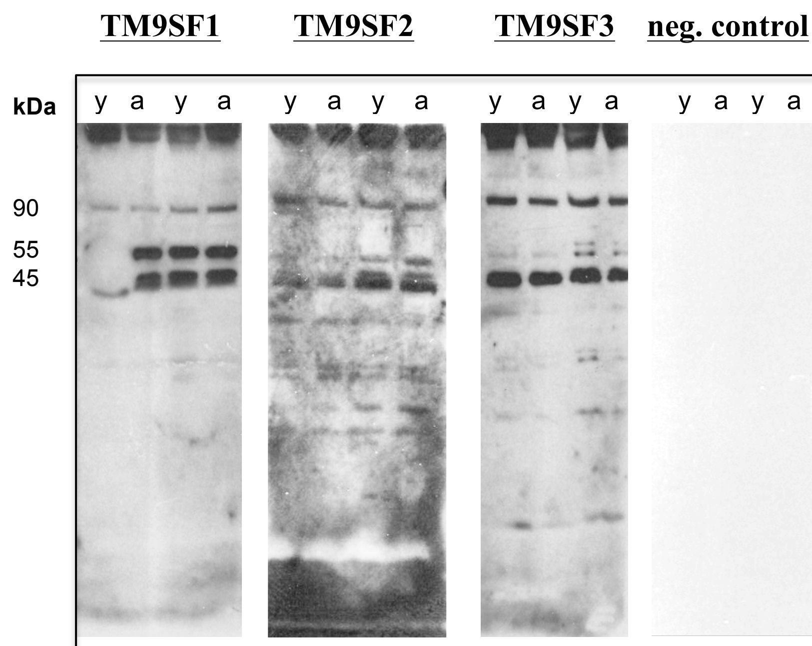


**Figure 4: Erythrocyte plasma membranes immunoblotted for the presence of arNOX proteins.** Processed erythrocyte plasma membranes of one young (y) and one aged (a) donor were immunostained with antibodies against TM9SF proteins. Each blot was loaded with single (5 µl, first two lanes) and double (10 µl, second two lanes) amounts of samples. TM9SF1-TM9SF3 are indicating the respective antibody used. Negative control (neg. control) was missing the primary antibody.

### 3.2.2 Analysis of erythrocytes for the presence of arNOX activity

Since we couldn't identify arNOX as an integral membrane protein in erythrocytes, we tried to at least detect its activity according to the method of Morré et al. (see chapter 2.3.4). For this purpose, blood of five aged (between 54 and 71 years) and five young (between 24 and 29 years) subjects of mixed gender was obtained from the Austrian Red Cross, and erythocytes isolated thereof were analysed for the presence of arNOX activity.

The average rate for total cytochrome c reduction was quite similar for both groups, young and aged, though there were of course small individual differences (figure 5A).

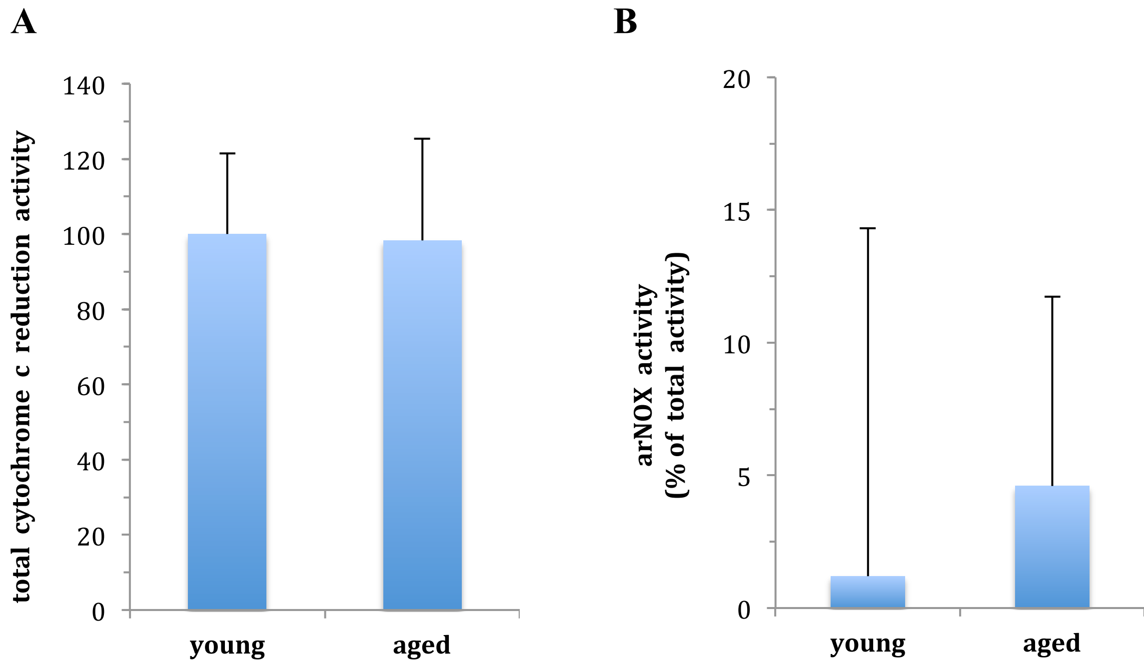


**Figure 5: arNOX activity measurement with red blood cells.** Erythrocytes of five young and five aged donors were analysed for cytochrome c reduction activity **(A)** in the absence of SOD - shown as total activity and expressed as *$(A_{550} - A_{540})$/min. $^{x}$ ml RBC* - and in parallel **(B)** in the presence of SOD. The proportion of cytochrome c reduction activity found to be SOD-inhibitable is referred to as arNOX activity. Mean values are shown, assigned with the corresponding standard deviation (± S.D.).

When SOD was added to remove the superoxide suggested to be produced by arNOX, the total cytochrome c reduction activity measured with RBCs of young and aged donors was hardly inhibited (figure 5B). On average, the proportion of SOD-inhibitable activity was 1 % for young subjects and nearly 5 % for aged subjects. However, all measurements involving SOD resulted in extremly high variations for obtained arNOX activity, even within duplicate samples. Maximal arNOX activity (namely 17 % of total cytochrome c reduction activity), for example, was obtained with a young subject, and one third of all measurements revealed negative arNOX activity, independent of age. Regarding the small number of subjects used for this study, the frequency of these evident inconsistencies is too prevalent to allow the conclusion that erythrocytes have arNOX activity and that this activity is elevated in aged subjects.

## 3.3 The plasma membrane redox system in erythrocytes

### 3.3.1 Gender-related differences in the reductive activities of red blood cells

Throughout previous experiments, we frequently observed that the reductive activities obtained with erythrocytes of males and females differed with the tendency to yield higher activities for female donors. To investigate the possible relevance of gender on transplasma membrane electron transports more detailed, blood of 18 females and 18 males was analysed. RBCs thereof were monitored for ferricyanide and cytochrome c reduction activity under identical conditions immediately after blood collection.

The latter activity was subdivided into haemolysis-independent and haemolysis-only proportions by using haemolysate standards (see chapter 3.1 for details). To exclude potential age-related effects, the age span of recruited participants was confined to range between 19 and 33 years only (figure 6).

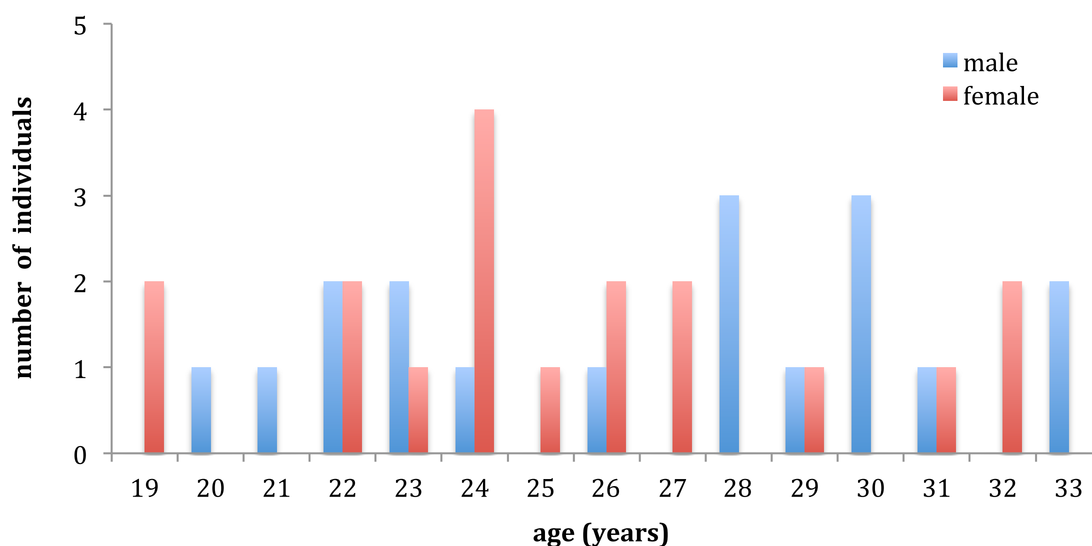


**Figure 6: Frequency distribution of subjects analysed, arranged according to their age and gender.** In total,18 males between 20 and 33 years and 18 females between 19 and 32 years participated in this study.

Results could confirm the already conceived suspicion that RBCs from females possess higher activities in plasma membrane electron transport than males.

In detail, females showed significantly elevated cytochrome c reduction activity, which here refers to the haemolysis-corrected proportion (figure 7A). The average increase was 18 % compared to males. When this activity wasn't corrected for haemolysis, the difference between males and females was reduced by 10 % and was no longer significant (data not shown).

Also, the rate of ferricyanide reduction clearly differed between both genders, yielding an average increase of 20 % with females (figure 7B). Both observations were statistically significant with p-values of 0.046 for cytochrome c reduction activity and 0.030 for ferricyanide reduction activity.

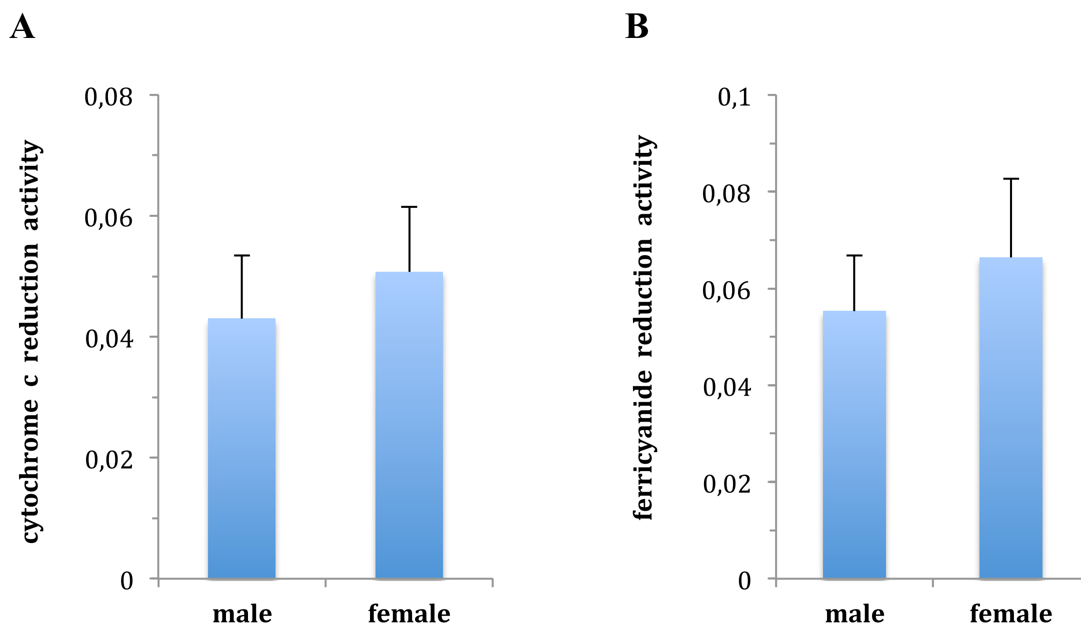


**Figure 7: Reductive activities of red blood cells from male and female donors. (A)** RBCs were analysed for the reduction of cytochrome c, the activity shown was corrected for haemolysis and expressed as *$(A_{550} - A_{540})$/min ˣ ml RBC*. The results of 15 males and 18 females are presented. Mean values are shown, assigned with the corresponding standard deviation (± S.D.). **(B)** Ferricyanide reduction activity was determined with RBCs from 16 males and 18 females, expressed as *nmol ferrocyanide/min ˣ µl RBC*. Mean values are shown, assigned with the corresponding standard deviation (± S.D.).

Interestingly, the ferricyanide reduction activity showed to increase with the age of female donors (r = 0.549) with high significance (p = 0.018). This correlation was not found for male donors, whose activities were distributed more horizontally over the whole age range (figure 8).

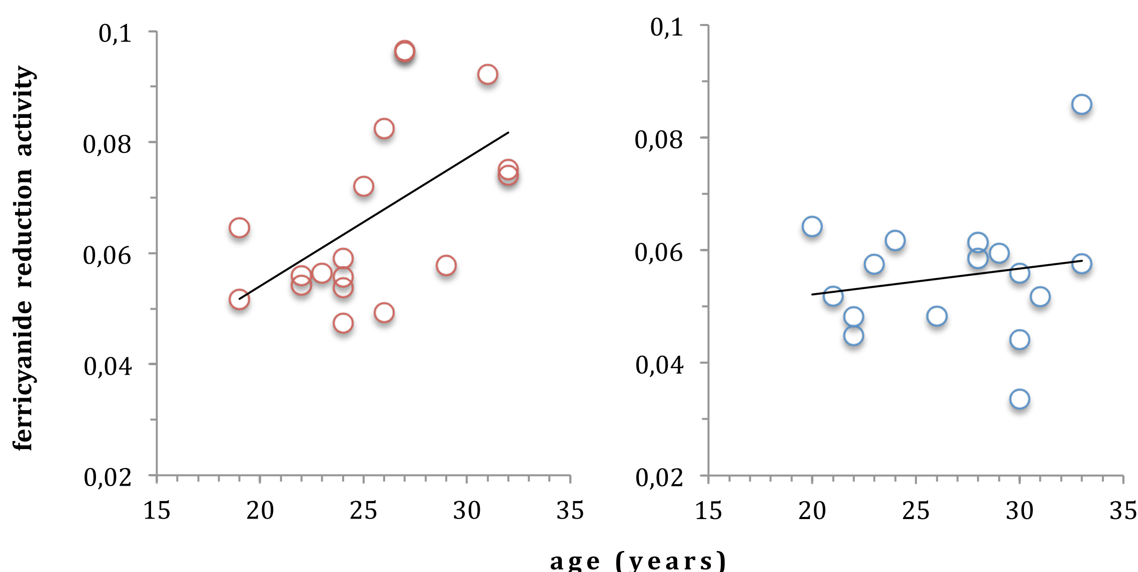


**Figure 8: Correlation between ferricyanide reduction activity and age of donors.** The analysis was performed with results of 18 female (red circles left) and 16 male donors (blue circles right). Activities shown are mean values of duplicate measurements, expressed as *nmol ferrocyanide/min* $^{x}$ *µl RBC*.

A significant positive correlation was also found when comparing the activities for ferricyanide and cytochrome c reduction obtained with female donors (r = 0.591; p = 0.01). Low activities measured with the one oxidant were mostly associated with low activities measured with the other oxidant and vice versa (figure 9). However, RBCs of male donors did not show a definite relation between the both differently determined reductive activities.

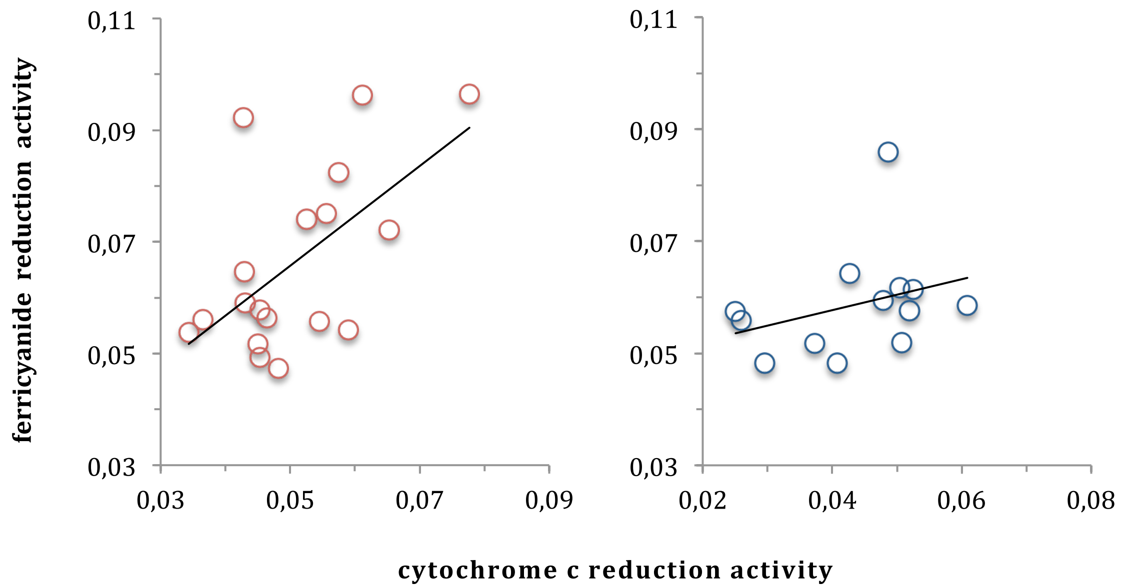


**Figure 9: Correlation between ferricyanide reduction and cytochrome c reduction activities.** The analysis was performed with results of 18 female (red circles left) and 13 male donors (blue circles right). Activities shown are mean values of duplicate measurements, expressed as *nmol ferrocyanide/min* $^x$ *µl RBC* and $(A_{550} - A_{540})/min$ $^x$ *ml RBC*, respectively. Cytochrome c reduction activity was corrected for haemolysis.

We finally wanted to see if there is any relationship between the reductive capacity of cytosol-associated constituents and the extracellular reduction of added oxidants. For this purpose, the activity of haemolysates were compared with the corresponding activities for ferricyanide and cytochrome c reduction obtained for each individual.

With females, the activities for haemolysates correlated very well and highly significantly with the amount of ferrocyanide produced (r = 0.667; p = 0.002), whereas no significant correlation was found for male donors (figure 10A). In contrast to that, the comparison of cytochrome c reduction activities with haemolysate activities did not yield a significant correlation for female donors (figure 10B). Here, however, the male's activities correlated quite well and were highly significant (r = 0.682; p = 0.005). These results would suggest a gender-specific connection of intracellular reservoirs of reductants with extracellularly measured reduction events, which remains to be investigated in more detail.

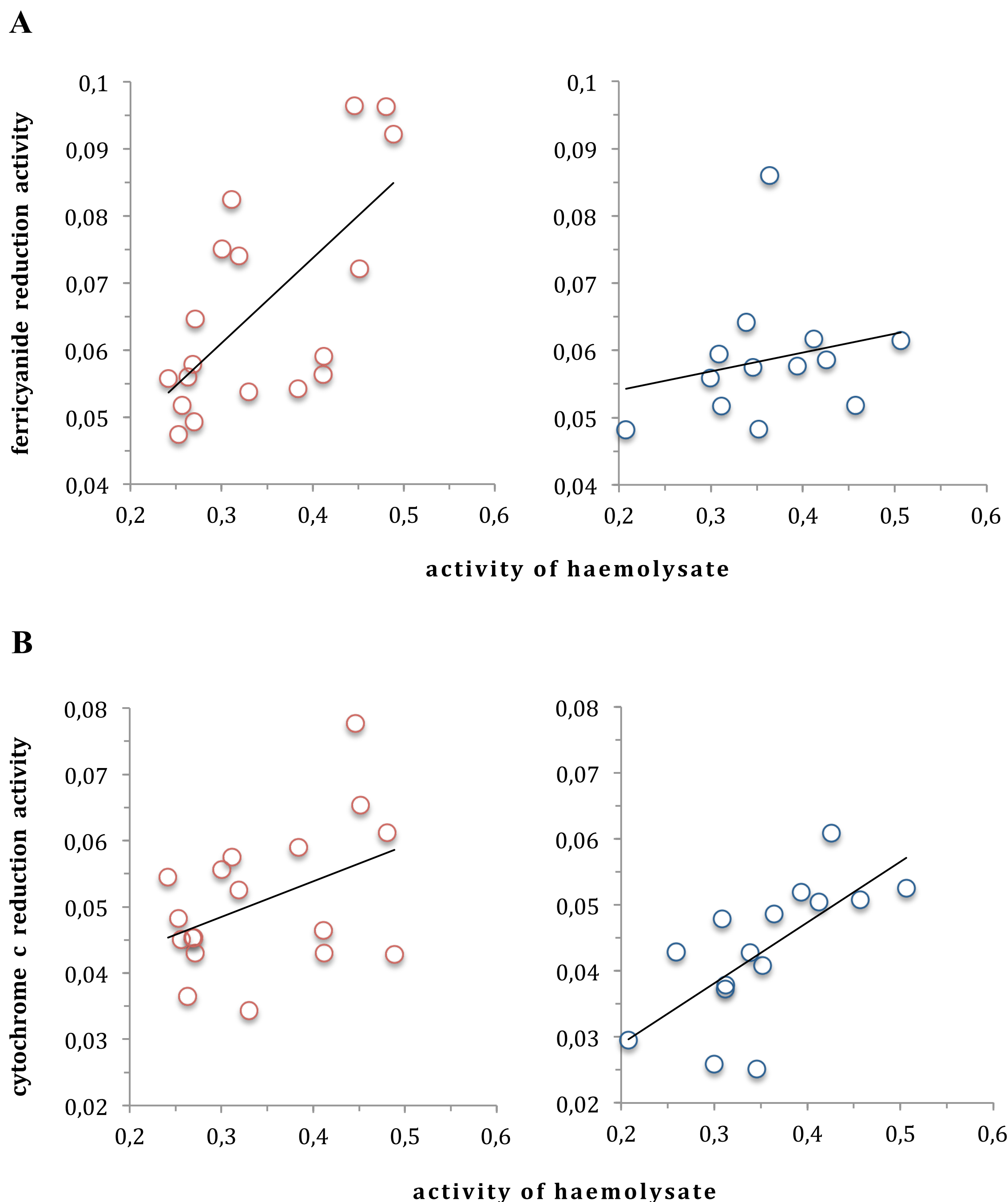


**Figure 10: Correlation of haemolysate activities with ferricyanide and cytochrome c reduction activities.** The activity for haemolysates was determined as described in chapter 3.1 and multiplied with the factor 1000 for demonstrational reasons only. **(A)** Analysis was performed with results of 18 female (red circles left) and 13 male donors (blue circles right). Activities are expressed as *nmol ferrocyanide/min* $^{x}$ *µl RBC* and *($A_{550}$ - $A_{540}$)/min* $^{x}$ *% haemolysis*, respectively. **(B)** Analysis included results of 18 female (red circles left) and 15 male donors (blue circles right). Cytochrome c reduction activity was corrected for haemolysis. Activities are expressed as *($A_{550}$ - $A_{540}$)/min* $^{x}$ *ml RBC* and *($A_{550}$ - $A_{540}$)/min* $^{x}$ *% haemolysis*, respectively. Mean values of duplicate measurements are shown.

### 3.3.2 The influence of selected treatment procedures on the plasma membrane electron transport activity of erythrocytes

To deepen the knowledge about the function and regulatory mechanisms of the plasma membrane electron transport system in human red blood blood cells, we applied selected treatment conditions and monitored changes in electron transport activity. These treatments included the chemicals cyclodextrin and vanadate, hyperglycaemic and hyperosmolaric conditions as well as loading cells with the antioxidant $CoQ_{10}$. Whenever possible, ferricyanide and cytochrome c reduction activities - the latter yet without haemolysis corrections, except for the blood storage assays - were analysed thereafter in parallel to see if one or the other activity was more affected.

#### *3.3.2.1 Hyperosmolarity*

We wanted to find out whether red blood cells modulate their transplasma electron transport system when adapting to osmolaric stress. For now, hypotonic conditions were excluded to prevent too much haemolysis, since we yet didn't know the reductive potential of released cytosolic components on ferricytochrome c and ferricyanide. So we focused on hyperosmolarity and increased the concentration of NaCl in the TBSG buffer used for preincubation and the reduction assays themselves (see chapter 2.4.4 for details).

Red blood cells significantly increased the electron transport through the plasma membrane in the hypertonic buffer. The average stimulation of reductive activities was plus 26 % for ferricyanide reduction and plus 70 % for cytochrome c reduction (figures 11A and 11B). The effect on the reduction rate of cytochrome c was analysed with RBCs of a male donor and was very reproducible throughout independently repeated measurements. Ferricyanide reduction analysis was performed with RBCs of a male and of a female, whereas the female's increase in activity upon hyperosmolaric treatment was about 3 times higher than that of the male. This effect might be interpreted as

gender-specific but can also have an individual background. Regrettably, we were not able to confirm these data because at that time, female blood donors were pretty rare.

#### *3.3.2.2 Hyperglycaemia*

Throughout all our experiments, isolated red blood cells were kept in buffers substituted with 7.5 mM glucose to maintain cell metabolism. According to the German Nutrition Society, this concentration would correspond to a postprandial blood sugar level of an average human. To test the influence of abnormal high glucose levels on the plasma membrane electron transport activity, RBCs of one male and one female were incubated with 12 mM glucose for hyperglycaemic conditions and with 2 mM glucose, whereas 2 mM glucose served as a control for hyperglycaemic effects.
With both subjects, we observed that hyperglycaemia slightly inhibited reductive activities (figures 11C and 11D). On average, ferricyanide reduction was slightly more affected (92 % of control activity) than cytochrome c reduction (96 % of control activity).

#### *3.3.2.3 Cyclodextrin*

The transfer of electrons from intracellular donors onto extracellular acceptors naturally involves the presence of intermediate electron acceptors (so-called electron carrier) in the plasma membrane. With the exception for coenzyme Q, these are yet fully uncharacterised but – similar to the electron transfer system in mitochondria - could involve further molecules and/or proteins, bound or freely floating in the viscous lipid bilayer (SUN *et al.* 1992). The fluidity and protein content of the plasma membrane is known to be dependent on its lipid composition, which can highly differ throughout the lipid bilayer, leading to the formation of so-called microdomains (MARGUET *et al.* 2006). Such microdomains are for example lipid rafts, which have relatively high amounts of cholesterol and sphingolipids and harbour specific proteins mostly involved in signalling events (BROWN 2002). The kind of lipids and their interactions with other components of the lipid bilayer therefore determine functional domains of the

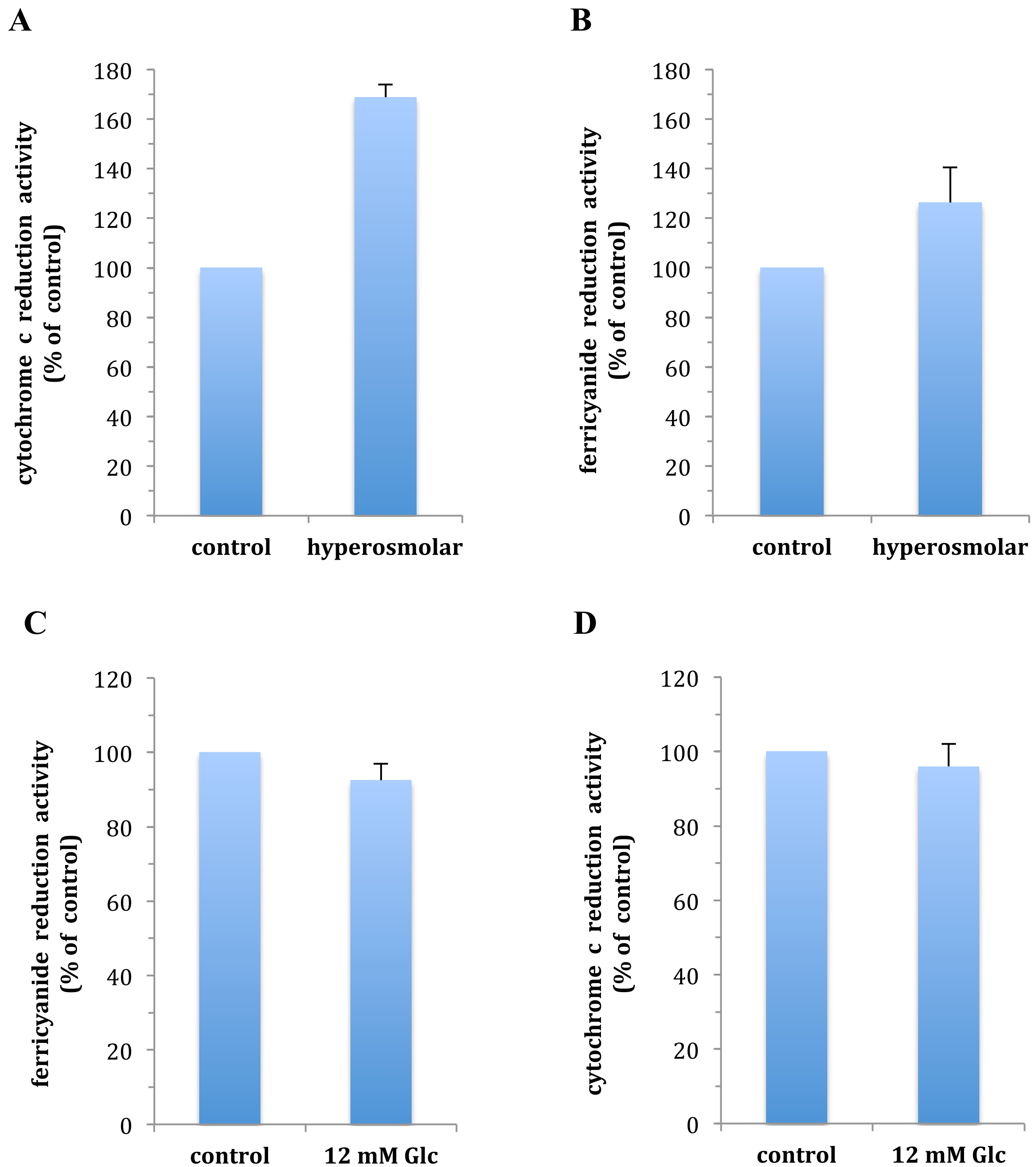


**Figure 11: Reductive capacity of RBCs kept under hyperosmotic stress and supplied with increased concentration of glucose. (A)** Red blood cells of one male (22 years) were analysed for cytochrome c reduction activity under normal (control) and hyperosmotic conditions. Mean values of three independent measurements are shown, assigned with the corresponding standard deviation (± S.D.). **(B)** Ferricyanide reduction activity analysis of the previous male and one female (32 years), whose RBCs were exposed to hyperosmotic stress. Mean values including both subjects analysed are shown, variations therein are indicated with an error bar.

**(C and D)** RBCs of a 56-year-old male and a 32-year-old female were monitored for the influence of glucose on ferricyanide and cytochrome c reduction activity, 12 mM (hyperglycaemic) and 2 mM glucose (control) were testet. Mean values including both subjects analysed are shown, variations therein are indicated with an error bar.

plasma membrane.

To alterate the natural lipid organisation in red blood cell cell membranes, methyl-ß-cyclodextrin was used due to its capability to bind to and seperate cholesterols from lipid bilayers (Zidovetzki and Levitan 2007). Cells were treated with 0.5 % and 0.25 % (which correspond to 4.4 mM and 2.2 mM) cyclodextrin for 20 min to achieve sufficient cholesterol depletion. The effect on transplasma electron transport was analysed for male and female donors as described in chapter 2.4.4.

The assay with 0.25 % cyclodextrin always revealed a slight increase for the ferricyanide-specific reductive activity analysed with both genders, which was on average 4 % of control activity (figure 12A). The same activity, in turn, remained quite unaffected or was even slightly reduced when the concentration of cyclodextrin was doubled to 0.5 %. Contrary to that, the activities for cytochrome c reduction obtained with two male donors were inconsistent. While increasing concentrations of cyclodextrin reduced the activity of the one male, they had an increasing stimulatory effect on the activity of the other (data not shown).

#### *3.3.2.4 Vanadate*

Vanadate is a popular and potent protein tyrosine phosphatase inhibitor but also affects the activity of several other phosphatases (Seargeant and Stinson 1979; Tessier *et al.* 1989). To investigate a possible connection between cellular dephosphorylation events and electron transport through the plasma membrane, red blood cells of three males and one female were treated with vanadate and subsequently assayed for the reduction of extracellular ferricyanide (see chapter 2.4.4. for details).

The reducing activity of male RBCs dropped on average for about 13 % with 50 µM vanadate, measured with ferricyanide, and this response was quite reproducible for all three subjects analysed (figure 12B). When analysing RBCs of one female, the rate of ferricyanide reduction increased to 115 % of control activity after treatment with the same concentration of vanadate (data not shown).

It remains to be investigated whether this distinct effect caused by vanadate occured

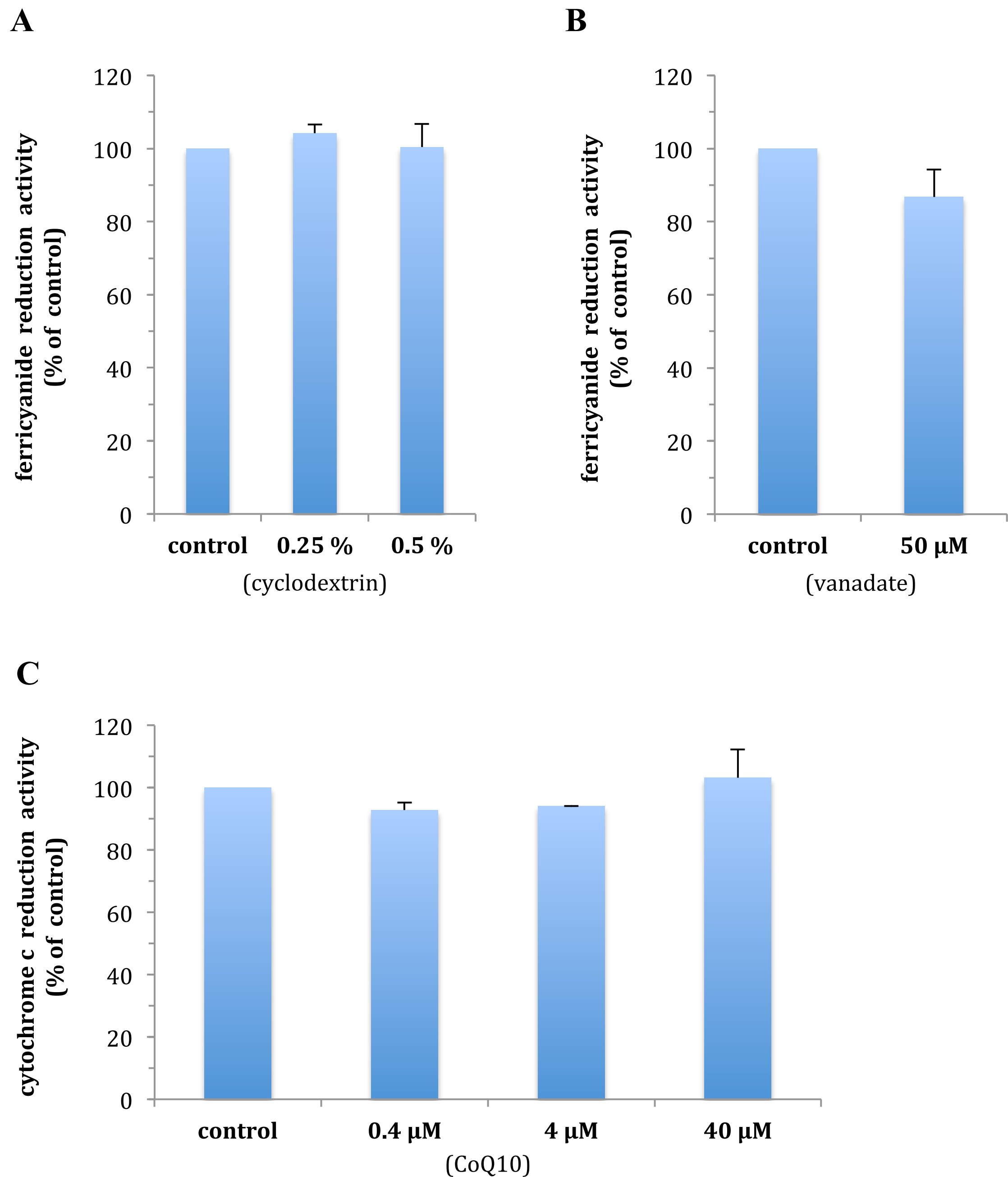


**Figure 12: Reductive capacity of RBCs treated with cyclodextrin and vanadate and supplied with coenzyme $Q_{10}$. (A)** Red blood cells of a 26-year-old male and a 32-year-old female were incubated with 0.25 % and 0.5 % cyclodextrin or left untreated (control). Reductive activity was analysed with ferricyanide. Mean values of four independent measurements (two for each subject) are shown, assigned with the corresponding standard deviation (± S.D.) **(B)** Analysis of male erythrocytes for the effect of vanadate on the plasma membrane electron transport using ferricyanide. Mean values of three independent measurements are shown, assigned with the corresponding standard deviation (± S.D.). **(C)** Coenzyme $Q_{10}$-preincubated RBCs of a 22-year-old male and a 23-year-old female were examined for cytochrome c reduction activity. Mean values including both subjects analysed are shown, variations therein are indicated with error bars.

due to gender-associated differences or are just the consequence of an individually regulated response, which was coincidentally not observed with the three males aforementioned.

#### *3.3.2.5 Coenzyme $Q_{10}$*

Coenzyme $Q_{10}$ ($CoQ_{10}$) is a biological antioxidant essentially contributing to a cell's healthiness and survival (LITTARRU and TIANO 2007). In the inner mitochondrial membrane, it serves as an important electron carrier for the generation of energy in the form of ATP, but it is also known to be enriched in other membranes of the cell, performing important antioxidative tasks (NAVAS *et al.* 2007). During the past recent years, its benefits in the prevention and treatment of diseases and in reducing aging-related cellular defects are intensively investigated and discussed (DHANASEKARAN and REN 2005; KIM *et al.* 2007). Today, $CoQ_{10}$ is already available in designer food, as a dietary supplement or in products of the cosmetic industry, always in conjunction with the auspicious prospect to stop aging. Formerly, the reduced form of $CoQ_{10}$, ubiquinol, was widely belived to solely possess the antioxidative capacity. However, several studies emphazised the unexpected additional antioxidative properties of the oxidised form, ubiquinone (PETILLO and HULTIN 2008).

In the following experiment, $CoQ_{10}$ in the form of ubiquinone was used to increase the antioxidative properties of erythrocytes. $CoQ_{10}$ is a quite big, hydrophobic compound and needs lipid carriers for cellular absorption, therefore, RBCs were incubated with ubiquinone-preloaded plasma (see chapter 2.4.4. for details). The antioxidative effect was monitored by measuring the extracellular reduction of cytochrome c.

Interestingly, the electron transport activity of RBCs seemed to first slightly decrease with increasing concentration of $CoQ_{10}$ (about 93 % of control activity), as shown for treatments with 0.4 µM and 4 µM of the antioxidant (figure 12C). With 40 µM $CoQ_{10}$, the activity returned back to a level comparable to untreated cells (103 % of control activity).

### 3.3.3 Storage-dependent variations in the plasma membrane electron transport of erythrocytes

During the first period of work, we were very happy to receive blood samples from the Austrian Red Cross. For safety reasons (analysing aliquotes for serious infectivity), these samples were already kept cool for at least one day by the organisation, so we had to deal with different storage conditions (up to six days) for each blood sample.

To test whether the time of blood storage has an effect on the plasma membrane electron transport activity of erythrocytes, we daily analysed one and the same blood sample over 5 days, starting at the day of blood collection.

Blood of four males, aged between 23 and 33 years, was used, and the reduction of ferricyanide and cytochrome c was monitored consecutively. Inbetween, the blood was stored at 4°C. For a more detailed insight, the cytochrome c reduction activity was corrected for haemolysis, also the activity per % haemolysis present was calculated (see chapter 3.1 for details).

As already assumed, the reductive activities varied over the time of four days storage (figure 13). Changes in activity seemed to occur rather randomly, days of higher or maximal activities and days of lower or minimal activities were different for each of the four subjects. Sometimes, the differently measured and expressed activities correlated quite well over all days (figure 13A), sometimes not or only partially (figure 13B). The diagrams include only two of the four males analysed, since they sufficiently represent the results of this experiment. As a consequence, further experiments were always performed with freshly collected blood to avoid storage-related misinterpretations when comparing RBCs of two individuals.

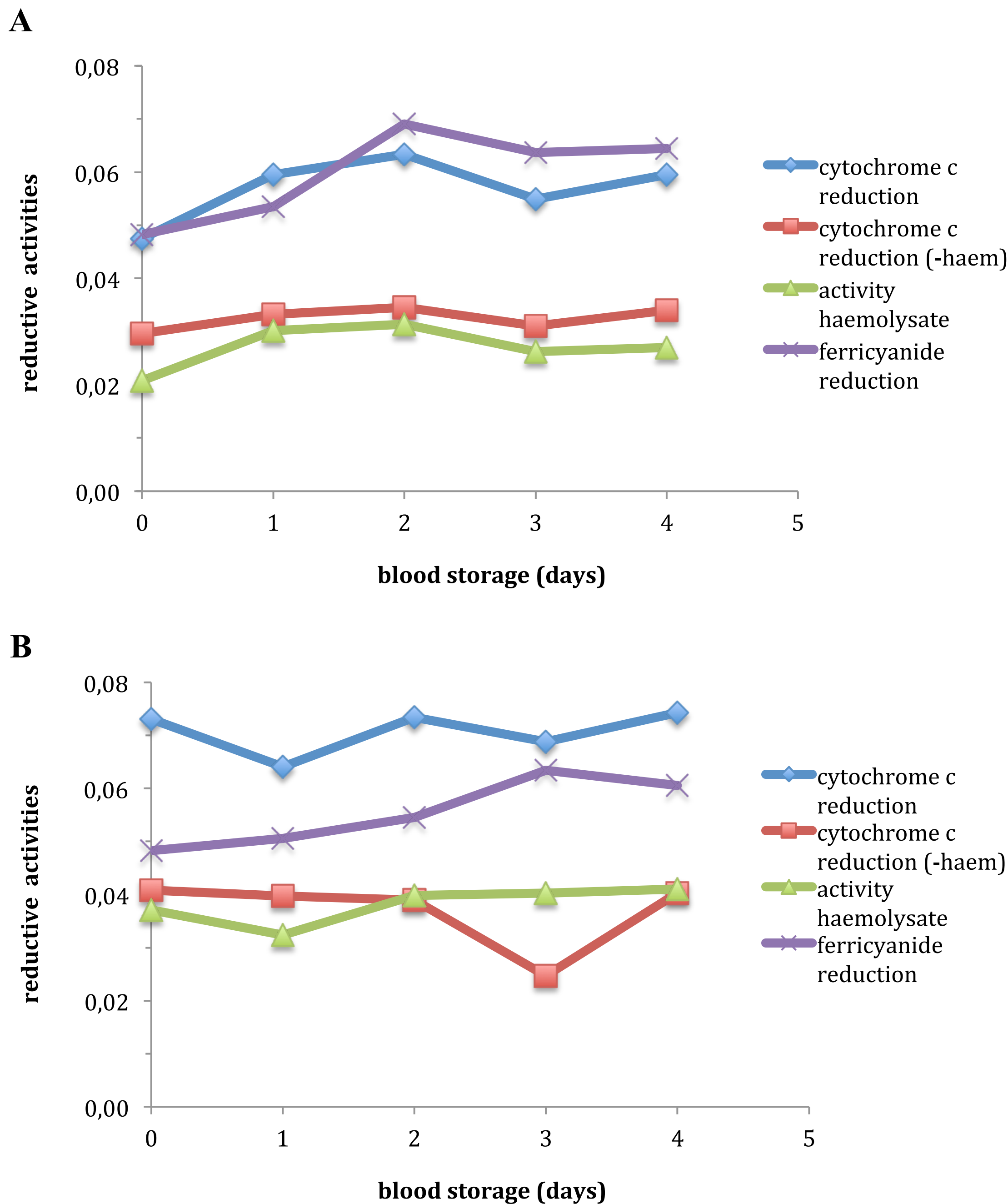


**Figure 13: Changes in the reductive capacity of RBCs over 4 days of blood storage.** RBCs of two males, 23 **(A)** and 26 years old **(B)**, were analysed for transmembrane electron transport activities immediately after blood collection (day 0) and on the following four days (days 1 to 4). The reduction of cytochrome c and ferricyanide was measured consecutively. The activity for cytochrome c reduction is shown as total activity obtained (cytochrome c reduction), the haemolysis-corrected activity thereof (-haem) and the activity resulting from haemolysis itself (activity haemolysate, expressed as *$(A_{550}$ - $A_{540})$/min.* $^{x}$ *% haemolysis* and multiplied with the factor 100 for illustrational reasons). Activities are expressed as *nmol ferrocyanide/min.* $^{x}$ *µl RBC* for ferricyanide and *$(A_{550}$ - $A_{540})$/min.* $^{x}$ *ml RBC* for cytochrome c reduction. Mean values of duplicate measurements are shown.

# 4 Discussion

The present study is based on spectrophotometrical measurements monitoring the reduction rates of ferricyanide and cytochrome c, whereas the first is a quite common oxidant for assaying transplasma membrane electron transport activities (AVRON and SHAVIT 1963; CLARK *et al.* 1981). Cytochrome c, or more specifically ferricytochrome c, was so far never employed before for this purpose - particularly with erythrocytes - as it usually is only used to indicate the presence and concentration of superoxide in biological samples (BABIOR *et al.* 1973; PRITCHARD *et al.* 1995; TARPEY *et al.* 1999). Since we anyway needed it for arNOX activity measurements (see next chapter), we thought to also include cytochrome c for the analysis of transplasma membrane electron transport activities as an alternative electron acceptor to ferricyanide. We could establish a method, which turned out to be at least as reliable as the one based on the reduction of ferricyanide and which was also applied in arNOX activity assays (see chapter 3.1).
Importantly, we were able to define the reduction potential of haemolysates, though only for the cytochrome c reduction method. It allowed us to calculate the amount of haemolysis occurring during assays and to determine cytochrome c reduction activities of intact cells more precisely. This improvement, however, was achieved in an advanced stage of our studies, so it was only applicable in later experiments, the blood storage and gender-specific analyses, to be exact.

## 4.1 arNOX was not detectable in whole cell and plasma membrane preparations of human erythrocytes

Our attempts to identify arNOX in red blood cells of aged human subjects failed. Neither its activity in intact cells nor the presence of the oxidase in membrane ghost preparations by western blot analysis could yield a definite result indicating a possible

association of arNOX with erythrocyte plasma membranes of aged donors (figures 4 and 5). It might be concluded that erythrocytes in general do not or only in non-detectable amounts possess this particular ECTO-NADH oxidase, but to really do so, two aspects concerning our methodical approaches have to be taken into consideration. First, the antibodies purchased where never shown to specifically recognise cell surface anchored oxidases since they were applied with sera and saliva samples, detecting only the truncated, shed forms of arNOX (TANG *et al.* 2013). Nevertheless, they were generated from N-terminal peptides of human TM9SF homologues and are therefore expected to bind also to non-soluble forms of arNOX, having large accessible extracellular N-terminal regions. However, to really confirm their explicitly useful application for the detection of both, the circulating and the membrane-associated forms of arNOX, these antibodies are suggested to undergo additional validations. Secondly, we were not able to identically reproduce the enzymatic activity assay Morré et al. were publishing, since we did not continuously record the reduction of ferricytochrome c but measured reductive activities only once at the beginning and once at the end of a 30 min incubation period (MORRE *et al.* 2008b). This was due to the fact that we included additional control samples (duplicates and negative controls), which needed to be analysed in parallel with the other samples to still serve as reliable controls. If we had analysed all these samples in the manner Morré et al. did, the time-consuming assay for only one sample would have required to prepare each single sample freshly from the same blood prior to the measurement. This in turn would implicate that controls were no veritable controls anymore since they then contained erythrocytes which were no longer prepared under truely identical conditions, regarding storage and isolation procedure. The inclusion of controls was considered necessary, as changes in the absorption measured were also seen with samples containing solely ferricytochrome c or red blood cells, which significantly contributed to the total activities obtained. Also the high variability in activity measured with duplicates suggested that these controls are indispensable (figure 5). It is easily conceivable that our methodological approach missed the periodically occurring burts of superoxide production by arNOX, so it is a strategy which

undoubtly needs to be improved to serve as a reliable method for the detection of age-related NADH oxidase activities.

## 4.2 Gender, hormones and their impact on the reductive potential of erythrocyte plasma membranes

Over decades of studying transplasma membrane electron transport systems, the aspect of gender-dependent differences has totally been neglected. However, these particular electron transfers are only part of a superordinated complex redox system, established in each cell to maintain a natural balance between essential reductive and oxidative events and to protect against oxidative damage (KEHRER and LUND 1994; KENNETT and KUCHEL 2003). Several studies were investigating gender- and sex hormone-related effects on selected key components - enzymes and antioxidants - of that redox system and clearly could show that sex matters (DEMIRBAG *et al.* 2005; MALORNI *et al.* 2007).
For example, the level of intracellular ascorbate, one of a cell's most abundant antioxidants, was shown to be modulated by the female sex hormone estrogen in brains of rats, with distinct effects. Certain brain regions of pubertal and adult females generally exhibited lower ascorbate levels compared to males of the same age, and the absence of estrogen during ovariectomy caused a significant increase in hippocampal ascorbate contents. On the other hand, females were less affected by ischaemia-induced decreases in brain ascorbate content, and estrogen treatment of ovariectomised ischaemic animals could reverse ascorbate contents to a level compareable with non-ovariectomised animals (KUME-KICK *et al.* 1996; KUME-KICK and RICE 1998). Estrogen has also been shown to increase the expression and activity of the antioxidative acting enzyme superoxide dismutase in cells of the murine vasculatur and in human leukocytes. This effect was antagonised by progesterone, another female sex hormone, leading to enhanced production of reactive oxygen species, which was also observed in rat brains after ovariectomy (SARAYMEN *et al.* 2003; WASSMANN *et al.* 2005). Further studies support the idea of estrogens as important hormonal antioxidants with

effects ranging from the prevention of lipid peroxidations to protecting neurons from oxidative stress, both being major risks for the emergence of cardiovascular diseases and neurological disorders (AYRES *et al.* 1996; BEHL *et al.* 1997).
Testosterone, the most abundant androgen in males, is thought to mediate resistance to oxidative stress, particularly in the cardiovascular system of males (MA and TONG 2010). Depriving testosterone by gonadectomy in male rats resulted in increased lipid peroxidations and production of ROS and dramatically reduced the level and activity of antioxidants like glutathion, superoxide dismutases and catalases in heart and prostate tissues (KLAPCINSKA *et al.* 2008; TAM *et al.* 2003).
The supplementation with testosterone after castration could partially restore the antioxidative capacity, though it was also shown to be able to further increase oxidative stress markers (KLAPCINSKA *et al.* 2008; PRUDOVA *et al.* 2007). Others are also supporting the prooxidative role of testosterone and its impact on mortality and diseases of males (ALONSO-ALVAREZ *et al.* 2007; AYDILEK *et al.* 2004; GARDNER-THORPE *et al.* 2003).
Regarding the multiple functions and the complex interplay and regulation of sex hormones throughout the human body and along maturation and aging, the influence of one hormone on a particular cell's or tissue's redox activities cannot be generalised and therefore has to be seen in a bigger context (BAIN 2007; JENSEN and DESOMBRE 1972).
Our findings that plasma membranes of female erythrocytes have significantly higher reductive capacities - based on the extracellular reduction of ferricyanide and ferricytochrome c - are indicative for the existence of a hormonal regulated redox system (figure 7). One factor for the female prevalence in reductive activity could be the intracellular level of ascorbate, which is thought to be a major cytosolic electron source for transplasma electron transports (VANDUIJN *et al.* 2000). As discussed earlier, ascorbate levels are assumed to be regulated by estrogen and, though males generally have higher intracellular ascorbate concentrations, the activity of ascorbate recycling could be increased in females (KUME-KICK and RICE 1998; MAURICE and LIGHTSEY 2007). Erythrocytes of females therefore might react more effectively to counteract extracellular oxidative stress. Whether the composition of redox enzymes and electron carriers in plasma membranes differs between genders, still needs to be claryfied but at

least has to be taken into consideration. Half of the women we analysed were regularly using hormonal contraceptions. However, there was no statistical difference in the reductive activities between females taking hormones and those which were not.

The ferricyanide reduction activity significantly increased with the age of females but not with that of males (figure 8). This probably indicates that female erythrocytes are also more capable to adapt to an ageing-related increase in oxidative stressing factors, though the relative differences in age of the subjects analysed are not reflecting aging in conventional manners (FINKEL and HOLBROOK 2000). However, our observation is consistent with results of several studies reporting that females possess higher amounts and activities for antioxidants and thus are less prone to oxidative damage during aging (ALI *et al.* 2006; BORRAS *et al.* 2003; GUEVARA *et al.* 2009). Interestingly, the reduction of cytochrome c did not correlate with the age of neither females nor males (data not shown). It might be that this activity is regulated differently in response to aging. Nevertheless, the transplasma membrane transfer of electrons onto ferricyanide and cytochrome c seems to be attributed to common mechanisms, at least in female erythrocytes, where these activities appeared to have a strong positive correlationship (figure 9). When comparing the reductive capacity of the cytosol with that obtained with whole cells, significant positive correlations were found, though in a gender-specific manner (figure 10). The cytosolic pool of potent reductants positively influenced ferricyanide reduction activity in female erythrocytes (figure 10A), whereas cytochrome c reduction activities remained rather unaffected (figure 10B).

This result may explain the earlier observed insensitivity of cytochrome c reduction activity with females for putative aging-triggered intracellular responses, which only affected ferricyanide reduction activity. With male erythrocytes, the reduction rate of ferricyanide seemed to be independent of the cytosolic reductive potential (figure 10A), which in contrast strongly affected cytochrome c reduction activity (figure 10B).

It is possible that these evident differences are achieved by gender-specific mechanisms, equipping the cytosol with particular antioxidants and other potential electron donors as well as controlling the accumulation and activity of plasma membrane-located participants of these electron transfer events. Importantly, activities of haemolysates

were quite similar for both genders (data not shown). It has to be noted that the reductive capacity of haemolysates was determined by cytochrome c reduction measurement, because the reduction of ferricyanide was insufficient for establishing a method to linearly correlate reductive activities with the percentage of haemolysis observed. Therefore, all the cytochrome c reduction discussed in this chapter refer to haemolysis-corrected activities, whereas ferricyanide reduction activities do not.

In summary, the dual analysis for ferricyanide and cytochrome c reduction suggests a common, but probably not identical system regulating both activities. Plasma membranes of female erythrocytes were shown to possess markedly increased reductive activities, proposed to serve as an antioxidative defense against extracellular accumulating oxidative stress. Contrary to males, ferricyanide reduction activity increased with age and was strongly associated with reductive capacities of the cytosol, which in turn affected reductive activities in a gender-specific manner.

It is of great importance to identify and characterise further membranous and cytosolic components contributing to plasma membrane electron transports in general as well as causing the gender-specific activity pattern we observed in the present study.

## 4.3 The transplasma membrane redox system of red bood cells is modulated in response to varying conditions

The conditions we were testing with red blood cells of various donors affected transplasma membrane redox activities to quite variable extents. Some effects were pretty evident and reproducible, whereas others were rather vague and marginal. An overview including all treatments and their resulting influences on reductive activities, insofar allowing a preliminary evaluation, is presented in table 6.

***Hyperosmolarity***

The hyperosmolaric buffer, erythrocytes were kept in throughout the whole experiment, particullarly increased the cytochrome c reduction activity with the male donor, up to 170 % of control samples, whereas the same donor's activity measured with ferricyanide was significantly less stimulated (figures 11A and 11B). As corresponding comprehensive analyses with females were not possible by that time, the noticeably high ferricyanide reduction rate of female RBCs in response to hyperosmolarity might indicate gender-related differences in the regulation of this activity but are so far lacking additional confirmation. The mechanisms underlying these effects are yet unknown and thus have to remain unspecified, but it is possible that, at least with males, this particular osmotic challenge caused disparities in the preference for reducing one or the other extracellular electron acceptor used.

***Hyperglycaemia***

The constant exposure of erythrocytes to 12 mM glucose throughout pretreatment and assay procedures resulted in a mild decrease of both reductive activities measured, independent of gender (figures 11C and 11D). This experiment was initally inspired by the study of Gray et al., who were investigating the effect of glucose on plasma membrane electron transports in pancreatic ß-cells of mice (GRAY *et al.* 2011). They were using similar concentrations than we did and could show that excess glucose significantly increased the rate of ferricyanide reduction to 300 % and even higher.
Since they preincubated cells up to 48 hours, we repeated experiments with prolonged glucose exposure (24 hours), but this resulted in substantial haemolysis, which persisted during activity measurement and made a reliable interpretation impossible (data not shown). We do not expect erythrocytes to respond similarly to glucose like pancreatic islet cells do, since the latter are specifically sensitive to changing levels of the sugar, known to modulate their production and secretion of insulin (MEGLASSON and MATSCHINSKY 1986). Glucose is an essential energy supplier for red blood cells, since they do not have mitochondria and thus are dependent on glycolysis for the generation of

ATP (CHAPMAN *et al.* 1962). Glycolysis, however, also produces NADH, an important reducing equivalent for transplasma membrane electron transfers (BERRIDGE and TAN 2000). We therefore conclude that the hyperglycaemic conditions probably induced other, yet uncharacterised processes in erythrocytes, causing the (provisorily) inhibitory profile observed for reductive activities. It would be interesting and certainly necessary to find out if glucose in milder concentrations revealed similar results.

***Cyclodextrin***

Cyclodextrin caused a concentration-dependent impact on the reductive activities of male and female erythrocytes, but with differing results for ferricyanide and cytochrome c reduction. While cytochrome c reduction activity was affected quite inconsistently - once positively, once negatively – (data not shown), the reduction rate of ferricyanide was initally mildly stimulated by mid-level concentrations of the compound, but dropped to control levels or somewhat below when the concentration of cyclodextrin exceeded 0.25 % (figure 12A).
ß-cyclodextrins are known to effectively and very specifically seperate cholesterols from erythrocyte plasma membranes without infiltrating the membrane and the cell itself. They were also reported to be involved in the removal of phospholipids and membrane proteins, the latter, though, to a far lesser extend than seen with cholesterols (GIOCONDI *et al.* 2004; OHTANI *et al.* 1989). Cholesterols are particularly enriched in lipid rafts, plasma membrane microdomains, which harbour essential proteins involved in cell signalling processes (BROWN 2002). Hence, the treatment with cyclodextrins can consequently result in alterations of cellular signal transduction events, as already demonstrated with T lymphocytes (KABOURIDIS *et al.* 2000). It has to be considered as well that the shifts of lipid composition in plasma membranes caused by cyclodextrin can increase the permeability of cells, thus faciliating the leakage of intracellular molecules and in the worst case promote cell death (KISS *et al.* 2010). However, all these effects discussed here were often very cell-type-specific and strongly depended on the concentration and incubation time applied.

The pattern of ferricyanide reduction activity in response to increasing concentrations of cyclodextrin we observed repeatedly could therefore be the result of multiple effects, which obviously require additional investigations to be defined in detail.
Currently, the diverging response of treated cells concerning cytochrome c reduction activity is so far inconclusive. Anyhow, individual factors contributing thereto cannot be excluded.

***Vanadate***

Vanadate is a phosphate analogue which competitively binds to phosphatases with varying affinity, depending on the type of phosphatase involved. Thereby vanadate prevents the enzymatic removal of phosphate groups from biological substrates (HUYER *et al.* 1997). It is commonly used for studying the function of protein tyrosine phosphatases since these enzymes are effectively inhibited by vanadate (GORDON 1991).
Our data indicated that vanadate has the potential to impair the transplasma membrane activity reducing ferricyanide, as revealed with RBCs of three males (figure 12B). Maximum inhibiton was likely already achieved with 50 µM vanadate, as a 20-fold concentration of the compound lead to nearly identical results and 10 µM vanadate was not sufficient to affect that activity (data not shown).
Many important cellular processes are dependent on protein phoshorylations since they are essential for the activation and propagation of signalling cascades. It is thus suggested that the inhibition of ferricyanide reduction activity seen with vanadate is the result of an overall impairment of cellular functions. This can also include enzymes of that particular redox system, but yet proof has to be brought forward.
There might exist a possible gender-specific response of treated erythrocytes to vanadate, since we obtained a converse effect, namely stimulated ferricyanide reduction activity, with one female (data not shown). However, this has still to be examined in more detail.

***Coenzyme $Q_{10}$***

The antioxidative properties of coenzyme $Q_{10}$ are widespread and crucial for the vitality of cells (LITTARRU and TIANO 2007). In addition, it exerts an essential role in plasma membrane redox systems by serving as electron carriers across the lipid bilayer and replenishing depleted reserves of other antioxidants (NAVAS *et al.* 2007; SUN *et al.* 1992). Therefore, the plasma membrane content of $CoQ_{10}$ is thought to greatly influence the activity of that system (NAVAS *et al.* 2007).

Surprisingly, our results revealed that it did not seem to stimulate but rather tended to inhibit redox activity, as measured by the reduction of cytochrome c, and even left it quite unaffected with the highest concentration of $CoQ_{10}$ applied (figure 12C). We are aware that these data are yet not sufficient to allow a more specific statement. However, it is possible that the amount of $CoQ_{10}$ taken up by cells was too small, either because it was not sufficiently solubilised by plasma lipids or because the concentrations used were too low. Since cells usually regulate the synthesis and distribution of the antioxidant themselves, exogenous supplementation is known to hardly influence intracellular concentrations of $CoQ_{10}$ (NIKLOWITZ *et al.* 2004).

The preincubation of ubiquinone with plasma, thus, might have been not enough and would have required the use of specific formulations of $CoQ_{10}$, which are known to faciliate its bioavailability (KUROWSKA *et al.* 2003). Also, it would have been interesting to find out if the redox response was similar when ferricyanide was used as the extracellular electron acceptor.

**Table 6: Summary of the effects observed with redox activities of erythrocytes upon respective treatments**. Stimulating and inhibiting effects, respectively, are indicated with **+** and **-**, respectively; n.d. = not definable. Symbols in brackets indicate slight trends, which, however, have to be investigated in more detail.

| Treatment procedure | Method of activity measurement | | Differences between genders |
|---|---|---|---|
| | Ferricyanide | Cytochrome c | |
| ***Hyperosmolarity*** | **+** | **+** | yes |
| ***Hyperglycaemia*** | **(-)** | **(-)** | no |
| ***Cyclodextrin*** | n.d. | / | no |
| ***Vanadate*** | **-** | / | yes |
| ***Coenzyme $Q_{10}$*** | / | **(-)** | no |

***Storage***

The daily analysis of red blood cells over a storage period of four days revealed valuable information about how individual redox activities are regulated in response to changes in temperature, nutrient and oxygen supply (figure 13). This was the reason to continue our experiments exclusively with freshly donated blood immediately after collection.

# 5 Conclusion

The present work could give strong indices that the transplasma membrane electron transport system of male and female erythrocytes is subject to different regulatory mechanism, probably caused by sex-specific hormones. Most of the conditions tested with red blood cells, like $CoQ_{10}$, glucose and cyclodextrin, are suggested to require a more detailed and improved approach to determine their impact on this redox system. For those which revealed relatively clear results, like hyperosmolarity and vanadate, it would be interesting to look more closely at the molecular mechanisms underlying these effects. However, our data presented herewith are only providing a glimpse on some of the factors which potentially influence the transplasma membrane redox system in human erythrocytes. It is certainly necessary to extend these analyses - particularly for treatment assays - by employing considerably increased numbers of blood samples, enabling us to reinforce or reject the effects we observed.

# 6 References

ALANO, C. C., W. YING and R. A. SWANSON, 2004 Poly(ADP-ribose) polymerase-1-mediated cell death in astrocytes requires NAD+ depletion and mitochondrial permeability transition. The Journal of biological chemistry **279:** 18895-18902.

ALI, S. S., C. XIONG, J. LUCERO, M. M. BEHRENS, L. L. DUGAN *et al.*, 2006 Gender differences in free radical homeostasis during aging: shorter-lived female C57BL6 mice have increased oxidative stress. Aging cell **5:** 565-574.

ALONSO-ALVAREZ, C., S. BERTRAND, B. FAIVRE, O. CHASTEL and G. SORCI, 2007 Testosterone and oxidative stress: the oxidation handicap hypothesis. Proceedings. Biological sciences / The Royal Society **274:** 819-825.

ARRIGONI, O., and M. C. DE TULLIO, 2002 Ascorbic acid: much more than just an antioxidant. Biochimica et biophysica acta **1569:** 1-9.

ASARD, H., N. HOREMANS and R. J. CAUBERGS, 1992 Transmembrane electron transport in ascorbate-loaded plasma membrane vesicles from higher plants involves a b-type cytochrome. FEBS letters **306:** 143-146.

ASHER, G., J. LOTEM, L. SACHS and Y. SHAUL, 2004 p53-dependent apoptosis and NAD(P)H:quinone oxidoreductase 1. Methods in enzymology **382:** 278-293.

ASKERLUND, P., and C. LARSSON, 1991 Transmembrane Electron Transport in Plasma Membrane Vesicles Loaded with an NADH-Generating System or Ascorbate. Plant physiology **96:** 1178-1184.

AVRON, M., and N. SHAVIT, 1963 A Sensitive and Simple Method for Determination of Ferrocyanide. Analytical biochemistry **6:** 549-554.

AYDILEK, N., M. AKSAKAL and A. Z. KARAKILCIK, 2004 Effects of testosterone and vitamin E on the antioxidant system in rabbit testis. Andrologia **36:** 277-281.

AYRES, S., M. TANG and M. T. SUBBIAH, 1996 Estradiol-17beta as an antioxidant: some distinct features when compared with common fat-soluble antioxidants. The Journal of laboratory and clinical medicine **128:** 367-375.

BABIOR, B. M., R. S. KIPNES and J. T. CURNUTTE, 1973 Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. The Journal of clinical investigation **52:** 741-744.

BACON, E., and D. J. MORRE, 2001 Plasma membrane NADH oxidase of maize roots responds to gravity and imposed centrifugal forces. Plant physiology and biochemistry : PPB / Societe francaise de physiologie vegetale **39:** 487-494.

BAIN, J., 2007 The many faces of testosterone. Clinical interventions in aging **2:** 567-576.

BAKER, M. A., D. J. LANE, J. D. LY, V. DE PINTO and A. LAWEN, 2004 VDAC1 is a transplasma membrane NADH-ferricyanide reductase. The Journal of biological chemistry **279:** 4811-4819.

BANHEGYI, G., L. BRAUN, M. CSALA, F. PUSKAS and J. MANDL, 1997 Ascorbate metabolism and its regulation in animals. Free radical biology & medicine **23:** 793-803.

BEDARD, K., and K. H. KRAUSE, 2007 The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiological reviews **87:** 245-313.

BEHL, C., T. SKUTELLA, F. LEZOUALC'H, A. POST, M. WIDMANN *et al.*, 1997 Neuroprotection against oxidative stress by estrogens: structure-activity relationship. Molecular pharmacology **51:** 535-541.

BELL-PEDERSEN, D., V. M. CASSONE, D. J. EARNEST, S. S. GOLDEN, P. E. HARDIN *et al.*, 2005 Circadian rhythms from multiple oscillators: lessons from diverse organisms. Nature reviews. Genetics **6:** 544-556.

BERCZI, A., K. M. FREDLUND and I. M. MOLLER, 1995 Purification and characterization of an NADH-hexacyanoferrate(III) reductase from spinach leaf plasma membrane. Archives of biochemistry and biophysics **320:** 65-72.

BERRIDGE, M. V., P. M. HERST and A. S. TAN, 2005 Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. Biotechnology Annual Review **11:** 127-152.

BERRIDGE, M. V., and A. S. TAN, 2000 Cell-surface NAD(P)H-oxidase: relationship to trans-plasma membrane NADH-oxidoreductase and a potential source of circulating NADH-oxidase. Antioxidants & redox signaling **2:** 277-288.

BORRAS, C., J. SASTRE, D. GARCIA-SALA, A. LLORET, F. V. PALLARDO *et al.*, 2003 Mitochondria from females exhibit higher antioxidant gene expression and lower oxidative damage than males. Free radical biology & medicine **34:** 546-552.

BRIGHTMAN, A. O., J. WANG, R. K. MIU, I. L. SUN, R. BARR *et al.*, 1992 A growth factor- and hormone-stimulated NADH oxidase from rat liver plasma membrane. Biochimica et biophysica acta **1105:** 109-117.

BROWN, D., 2002 Structure and function of membrane rafts. International journal of medical microbiology : IJMM **291:** 433-437.

BROWN, D. I., and K. K. GRIENDLING, 2009 Nox proteins in signal transduction. Free radical biology & medicine **47:** 1239-1253.

BRUNO, M., A. O. BRIGHTMAN, J. LAWRENCE, D. WERDERITSH, D. M. MORRE *et al.*, 1992 Stimulation of NADH oxidase activity from rat liver plasma membranes by growth factors and hormones is decreased or absent with hepatoma plasma membranes. The Biochemical journal **284 ( Pt 3):** 625-628.

BUTLER, J., W. H. KOPPENOL and E. MARGOLIASH, 1982 Kinetics and mechanism of the reduction of ferricytochrome c by the superoxide anion. The Journal of biological chemistry **257:** 10747-10750.

CHAPMAN, R. G., M. A. HENNESSEY, A. M. WALTERSDORPH, F. M. HUENNEKENS and B. W. GABRIO, 1962 Erythrocyte metabolism. V. Levels of glycolytic enzymes and regulation of glycolysis. The Journal of clinical investigation **41:** 1249-1256.

CHLUBA-DE TAPIA, J., M. DE TAPIA, V. JAGGIN and A. N. EBERLE, 1997 Cloning of a human multispanning membrane protein cDNA: evidence for a new protein family. Gene **197:** 195-204.

CHUEH, P. J., C. KIM, N. CHO, D. M. MORRE and D. J. MORRE, 2002 Molecular cloning and characterization of a tumor-associated, growth-related, and time-keeping hydroquinone (NADH) oxidase (tNOX) of the HeLa cell surface. Biochemistry **41:** 3732-3741.

CHUEH, P. J., D. M. MORRE, C. PENEL, T. DEHAHN and D. J. MORRE, 1997 The hormone-responsive NADH oxidase of the plant plasma membrane has properties of a NADH:protein disulfide reductase. The Journal of biological chemistry **272:** 11221-11227.

CIMEN, M. Y., 2008 Free radical metabolism in human erythrocytes. Clinica chimica acta; international journal of clinical chemistry **390:** 1-11.

CLARK, M. G., E. J. PARTICK, G. S. PATTEN, F. L. CRANE, H. LOW *et al.*, 1981 Evidence for the extracellular reduction of ferricyanide by rat liver. A trans-plasma membrane redox system. The Biochemical journal **200:** 565-572.

CLULEY, H. J., and E. J. NEWMAN, 1963 The determination of small amounts of iron. Some aspects of new colorimetric methods. Analyst **88:** 3-17.

COLAVITTI, R., and T. FINKEL, 2005 Reactive oxygen species as mediators of cellular senescence. IUBMB life **57:** 277-281.

COLOMBINI, M., 1980 Structure and mode of action of a voltage dependent anion-selective channel (VDAC) located in the outer mitochondrial membrane. Annals of the New York Academy of Sciences **341:** 552-563.

CROSS, A. R., and A. W. SEGAL, 2004 The NADPH oxidase of professional phagocytes--prototype of the NOX electron transport chain systems. Biochimica et biophysica acta **1657:** 1-22.

CUTLER, R. G., 1992 Genetic stability and oxidative stress: common mechanisms in aging and cancer. EXS **62:** 31-46.

DAVIES, K. J., 1995 Oxidative stress: the paradox of aerobic life. Biochemical Society symposium **61:** 1-31.

DAVIES, S. L., and J. BOZZO, 2006 Spotlight on tNOX: a tumor-selective target for cancer therapies. Drug news & perspectives **19:** 223-225.

DEL PRINCIPE, D., L. AVIGLIANO, I. SAVINI and M. V. CATANI, 2011 Trans-plasma membrane electron transport in mammals: functional significance in health and disease. Antioxidants & redox signaling **14:** 2289-2318.

DEMIRBAG, R., R. YILMAZ and O. EREL, 2005 The association of total antioxidant capacity with sex hormones. Scandinavian cardiovascular journal : SCJ **39:** 172-176.

DHANASEKARAN, M., and J. REN, 2005 The emerging role of coenzyme Q-10 in aging, neurodegeneration, cardiovascular disease, cancer and diabetes mellitus. Current neurovascular research **2:** 447-459.

DICK, S. S., A. RYUZOJI, D. M. MORRE and D. J. MORRE, 2013 Identification of the constitutive ultradian oscillator of the circadian clock (ENOX1) in Saccharomyces cerevisiae. Advances in Biological Chemistry **3:** 320-328.

EMERIT, J., M. EDEAS and F. BRICAIRE, 2004 Neurodegenerative diseases and oxidative stress. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie **58:** 39-46.

ESTERBAUER, H., G. WAG and H. PUHL, 1993 Lipid peroxidation and its role in atherosclerosis. British medical bulletin **49:** 566-576.

FINKEL, T., and N. J. HOLBROOK, 2000 Oxidants, oxidative stress and the biology of ageing. Nature **408:** 239-247.

GARDNER-THORPE, D., C. O'HAGEN, I. YOUNG and S. J. LEWIS, 2003 Dietary supplements of soya flour lower serum testosterone concentrations and improve markers of oxidative stress in men. European journal of clinical nutrition **57:** 100-106.

GIOCONDI, M. C., P. E. MILHIET, P. DOSSET and C. LE GRIMELLEC, 2004 Use of cyclodextrin for AFM monitoring of model raft formation. Biophysical journal **86:** 861-869.

GOLDENBERG, H., H. LANDERTSHAMER and H. LAGGNER, 2000 Functions of vitamin C as a mediator of transmembrane electron transport in blood cells and related cell culture models. Antioxidants & redox signaling **2:** 189-196.

GOMEZ-DIAZ, C., J. C. RODRIGUEZ-AGUILERA, M. P. BARROSO, J. M. VILLALBA, F. NAVARRO *et al.*, 1997 Antioxidant ascorbate is stabilized by NADH-coenzyme Q10 reductase in the plasma membrane. Journal of bioenergetics and biomembranes **29:** 251-257.

GONZALEZ-GRONOW, M., T. KALFA, C. E. JOHNSON, G. GAWDI and S. V. PIZZO, 2003 The voltage-dependent anion channel is a receptor for plasminogen kringle 5 on human endothelial cells. The Journal of biological chemistry **278:** 27312-27318.

GORDON, J. A., 1991 Use of vanadate as protein-phosphotyrosine phosphatase inhibitor. Methods in enzymology **201:** 477-482.

GRAY, J. P., T. EISEN, G. W. CLINE, P. J. SMITH and E. HEART, 2011 Plasma membrane electron transport in pancreatic beta-cells is mediated in part by NQO1. American journal of physiology. Endocrinology and metabolism **301:** E113-121.

GRAY, S. P., E. DI MARCO, J. OKABE, C. SZYNDRALEWIEZ, F. HEITZ *et al.*, 2013 NADPH oxidase 1 plays a key role in diabetes mellitus-accelerated atherosclerosis. Circulation **127:** 1888-1902.

GREBING, C., F. L. CRANE, H. LOW and K. HALL, 1984 A transmembranous NADH-dehydrogenase in human erythrocyte membranes. Journal of bioenergetics and biomembranes **16:** 517-533.

GRIECO, P. A., and D. J. MORRE, 1999 Glaucarubolone and Simalikalactone D, respectively, preferentially inhibit auxin-induced and constitutive Components of Plant Cell Enlargement and the Plasma Membrane NADH Oxidase. International Journal of Plant Science **160:** 291-297.

GRIM, J., P. JANDIK, I. SLANSKA, E. DOLEZALOVA-BRCAKOVA, L. FUKSA *et al.*, 2012 Low expression of NQO1 predicts pathological complete response to neoadjuvant chemotherapy in breast cancer patients treated with TAC regimen. Folia biologica **58:** 185-192.

GUEVARA, R., F. M. SANTANDREU, A. VALLE, M. GIANOTTI, J. OLIVER *et al.*, 2009 Sex-dependent differences in aged rat brain mitochondrial function and oxidative stress. Free radical biology & medicine **46:** 169-175.

HOREMANS, N., H. ASARD and R. J. CAUBERGS, 1994 The Role of Ascorbate Free Radical as an Electron Acceptor to Cytochrome b-Mediated Trans-Plasma Membrane Electron Transport in Higher Plants. Plant physiology **104:** 1455-1458.

HULTQUIST, D. E., and P. G. PASSON, 1971 Catalysis of methaemoglobin reduction by erythrocyte cytochrome B5 and cytochrome B5 reductase. Nature: New biology **229:** 252-254.

HUYER, G., S. LIU, J. KELLY, J. MOFFAT, P. PAYETTE *et al.*, 1997 Mechanism of inhibition of protein-tyrosine phosphatases by vanadate and pervanadate. The Journal of biological chemistry **272:** 843-851.

HYUN, D. H., S. S. EMERSON, D. G. JO, M. P. MATTSON and R. DE CABO, 2006 Calorie restriction up-regulates the plasma membrane redox system in brain cells and suppresses oxidative stress during aging. Proceedings of the National Academy of Sciences of the United States of America **103:** 19908-19912.

IDO, Y., 2007 Pyridine nucleotide redox abnormalities in diabetes. Antioxidants & redox signaling **9:** 931-942.

JENSEN, E. V., and E. R. DESOMBRE, 1972 Mechanism of action of the female sex hormones. Annual review of biochemistry **41:** 203-230.

JEYA, M., H. J. MOON, J. L. LEE, I. W. KIM and J. K. LEE, 2010 Current state of coenzyme Q(10) production and its applications. Applied microbiology and biotechnology **85:** 1653-1663.

JIANG, Z., N. M. GORENSTEIN, D. M. MORRE and D. J. MORRE, 2008 Molecular cloning and characterization of a candidate human growth-related and time-keeping constitutive cell surface hydroquinone (NADH) oxidase. Biochemistry **47:** 14028-14038.

KABOURIDIS, P. S., J. JANZEN, A. L. MAGEE and S. C. LEY, 2000 Cholesterol depletion disrupts lipid rafts and modulates the activity of multiple signaling pathways in T lymphocytes. European journal of immunology **30:** 954-963.

KEHRER, J. P., and L. G. LUND, 1994 Cellular reducing equivalents and oxidative stress. Free radical biology & medicine **17:** 65-75.

KENNETT, E. C., and P. W. KUCHEL, 2003 Redox reactions and electron transfer across the red cell membrane. IUBMB Life **55:** 375-385.

KERN, D. G., Z. D. DRAELOS, C. MEADOWS, D. JAMES MORRE and D. M. MORRE, 2010 Controlling reactive oxygen species in skin at their source to reduce skin aging. Rejuvenation research **13:** 165-167.

KEYES, S. R., and D. L. CINTI, 1980 Biochemical properties of cytochrome b5-dependent microsomal fatty acid elongation and identification of products. The Journal of biological chemistry **255:** 11357-11364.

KIM, D. W., I. K. HWANG, K. Y. YOO, C. K. WON, W. K. MOON *et al.*, 2007 Coenzyme Q_{10} effects on manganese superoxide dismutase and glutathione peroxidase in the hairless mouse skin induced by ultraviolet B irradiation. BioFactors **30:** 139-147.

KISHI, T., D. M. MORRE and D. J. MORRE, 1999 The plasma membrane NADH oxidase of HeLa cells has hydroquinone oxidase activity. Biochimica et biophysica acta **1412:** 66-77.

KISS, T., F. FENYVESI, I. BACSKAY, J. VARADI, E. FENYVESI *et al.*, 2010 Evaluation of the cytotoxicity of beta-cyclodextrin derivatives: evidence for the role of cholesterol extraction. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences **40:** 376-380.

KLAPCINSKA, B., S. JAGSZ, E. SADOWSKA-KREPA, J. GORSKI, K. KEMPA *et al.*, 2008 Effects of castration and testosterone replacement on the antioxidant defense system in rat left ventricle. The journal of physiological sciences : JPS **58:** 173-177.

KOCH-NOLTE, F., F. HAAG, A. H. GUSE, F. LUND and M. ZIEGLER, 2009 Emerging roles of NAD+ and its metabolites in cell signaling. Science signaling **2:** mr1.

KUME-KICK, J., D. C. FERRIS, I. RUSSO-MENNA and M. E. RICE, 1996 Enhanced oxidative stress in female rat brain after gonadectomy. Brain research **738:** 8-14.

KUME-KICK, J., and M. E. RICE, 1998 Estrogen-dependent modulation of rat brain ascorbate levels and ischemia-induced ascorbate loss. Brain research **803:** 105-113.

KUROWSKA, E. M., G. DRESSER, L. DEUTSCH, E. BASSOO and D. J. FREEMAN, 2003 Relative bioavailability and antioxidant potential of two coenzyme q10 preparations. Annals of nutrition & metabolism **47:** 16-21.

LARM, J. A., F. VAILLANT, A. W. LINNANE and A. LAWEN, 1994 Up-regulation of the plasma membrane oxidoreductase as a prerequisite for the viability of human Namalwa rho 0 cells. The Journal of biological chemistry **269:** 30097-30100.

LATUNDE-DADA, G. O., J. VAN DER WESTHUIZEN, C. D. VULPE, G. J. ANDERSON, R. J. SIMPSON *et al.*, 2002 Molecular and functional roles of duodenal cytochrome B (Dcytb) in iron metabolism. Blood cells, molecules & diseases **29:** 356-360.

LAWEN, A., R. D. MARTINUS, G. L. MCMULLEN, P. NAGLEY, F. VAILLANT *et al.*, 1994 The universality of bioenergetic disease: the role of mitochondrial mutation and the putative inter-relationship between mitochondria and plasma membrane NADH oxidoreductase. Molecular aspects of medicine **15 Suppl:** s13-27.

LEE, H. C., and Y. H. WEI, 2009 Mitochondrial DNA instability and metabolic shift in human cancers. International journal of molecular sciences **10:** 674-701.

LI, R., M. A. BIANCHET, P. TALALAY and L. M. AMZEL, 1995 The three-dimensional structure of NAD(P)H:quinone reductase, a flavoprotein involved in cancer chemoprotection and chemotherapy: mechanism of the two-electron reduction. Proceedings of the National Academy of Sciences of the United States of America **92:** 8846-8850.

LITTARRU, G. P., and L. TIANO, 2007 Bioenergetic and antioxidant properties of coenzyme Q10: recent developments. Molecular biotechnology **37:** 31-37.

LLOYD, D., and F. KIPPERT, 1993 Intracellular coordination by the ultradian clock. Cell biology international **17:** 1047-1052.

LUSTER, D. G., and T. J. BUCKHOUT, 1989 Purification and Identification of a Plasma Membrane Associated Electron Transport Protein from Maize (Zea mays L.) Roots. Plant physiology **91:** 1014-1019.

MA, R. C., and P. C. TONG, 2010 Testosterone levels and cardiovascular disease. Heart **96:** 1787-1788.

MALORNI, W., I. CAMPESI, E. STRAFACE, S. VELLA and F. FRANCONI, 2007 Redox features of the cell: a gender perspective. Antioxidants & redox signaling **9:** 1779-1801.

MARGUET, D., P. F. LENNE, H. RIGNEAULT and H. T. HE, 2006 Dynamics in the plasma membrane: how to combine fluidity and order. The EMBO journal **25:** 3446-3457.

MAURICE, D. V., and S. F. LIGHTSEY, 2007 Sexual difference in ascorbic acid synthesis, tissue ascorbic acid and plasma total antioxidant capacity in mature chickens. British poultry science **48:** 519-523.

MAY, J. M., Z. QU and J. D. MORROW, 2001 Mechanisms of ascorbic acid recycling in human erythrocytes. Biochimica et biophysica acta **1528:** 159-166.

MAY, J. M., Z. C. QU and C. E. COBB, 2004 Human erythrocyte recycling of ascorbic acid: relative contributions from the ascorbate free radical and dehydroascorbic acid. J Biol Chem **279:** 14975-14982.

MAY, J. M., Z. C. QU and R. R. WHITESELL, 1995a Ascorbate is the major electron donor for a transmembrane oxidoreductase of human erythrocytes. Biochimica et biophysica acta **1238:** 127-136.

MAY, J. M., Z. C. QU and R. R. WHITESELL, 1995b Ascorbic acid recycling enhances the antioxidant reserve of human erythrocytes. Biochemistry **34:** 12721-12728.

MCKIE, A. T., D. BARROW, G. O. LATUNDE-DADA, A. ROLFS, G. SAGER *et al.*, 2001 An iron-regulated ferric reductase associated with the absorption of dietary iron. Science **291:** 1755-1759.

MEGLASSON, M. D., and F. M. MATSCHINSKY, 1986 Pancreatic islet glucose metabolism and regulation of insulin secretion. Diabetes/metabolism reviews **2:** 163-214.

MENDIRATTA, S., Z. C. QU and J. M. MAY, 1998 Erythrocyte ascorbate recycling: antioxidant effects in blood. Free radical biology & medicine **24:** 789-797.

MITCHISON, J. M., 2003 Growth during the cell cycle. International review of cytology **226:** 165-258.

MIURA, Y., and T. ENDO, 2010 Survival responses to oxidative stress and aging. Geriatrics & gerontology international **10 Suppl 1:** S1-9.

MORRE, D. J., 1994 Hormone- and growth factor-stimulated NADH oxidase. Journal of bioenergetics and biomembranes **26:** 421-433.

MORRE, D. J., 1995 NADH oxidase activity of HeLa plasma membranes inhibited by the antitumor sulfonylurea N-(4-methylphenylsulfonyl)-N'-(4-chlorophenyl) urea (LY181984) at an external site. Biochimica et biophysica acta **1240:** 201-208.

MORRE, D. J., 1998 NADH oxidase: A multifunctional ectoprotein of the eukaryotic cell surface. Plasma Membrane Redox Systems and their Role in Biological Stress and Disease**:** 121–156.

MORRE, D. J., P. J. CHUEH and D. M. MORRE, 1995a Capsaicin inhibits preferentially the NADH oxidase and growth of transformed cells in culture. Proceedings of the National Academy of Sciences of the United States of America **92:** 1831-1835.

MORRE, D. J., P. J. CHUEH, J. PLETCHER, X. TANG, L. Y. WU *et al.*, 2002a Biochemical basis for the biological clock. Biochemistry **41:** 11941-11945.

MORRE, D. J., B. HOSTETLER, N. WESTON, C. KIM and D. M. MORRE, 2008a Cancer type-specific tNOX isoforms: A putative family of redox protein splice variants with cancer diagnostic and prognostic potential. BioFactors **34:** 201-207.

MORRE, D. J., J. LAWLER, S. WANG, T. W. KEENAN and D. M. MORRE, 2002b Entrainment in solution of an oscillating NADH oxidase activity from the bovine milk fat globule membrane with a temperature-compensated period length suggestive of an ultradian time-keeping (clock) function. Biochimica et biophysica acta **1559:** 10-20.

MORRE, D. J., and D. M. MORRE, 1995 Differential response of the NADH oxidase of plasma membranes of rat liver and hepatoma and HeLa cells to thiol reagents. Journal of bioenergetics and biomembranes **27:** 137-144.

MORRE, D. J., and D. M. MORRE, 2003a Cell surface NADH oxidases (ECTO-NOX proteins) with roles in cancer, cellular time-keeping, growth, aging and neurodegenerative diseases. Free radical research **37:** 795-808.

MORRE, D. J., and D. M. MORRE, 2006a Aging-related cell surface ECTO-NOX protein, arNOX, a preventive target to reduce atherogenic risk in the elderly. Rejuvenation research **9:** 231-236.

MORRE, D. J., and D. M. MORRE, 2006b Membrane redox as an essential component of how cells increase in size following cell division. Acta Biologica Szegediensis **50:** 75-77.

MORRE, D. J., and D. M. MORRE, 2013 ECTO-NOX Proteins. 261-311.

MORRE, D. J., D. M. MORRE and P. TERNES, 2003a Auxin-activated NADH oxidase activity of soybean plasma membranes is distinct from the constitutive plasma membrane NADH oxidase and exhibits prion-like properties. In vitro cellular & developmental biology. Plant : journal of the Tissue Culture Association **39:** 368-376.

MORRE, D. J., R. POGUE and D. M. MORRE, 2001 Soybean cell enlargement oscillates with a temperature-compensated period length of ca. 24 min. In vitro cellular & developmental biology. Plant : journal of the Tissue Culture Association **37:** 19-23.

MORRE, D. J., E. SUN, C. GEILEN, L. Y. WU, R. DE CABO *et al.*, 1996 Capsaicin inhibits plasma membrane NADH oxidase and growth of human and mouse melanoma lines. European journal of cancer **32A:** 1995-2003.

MORRE, D. J., L. Y. WU and D. M. MORRE, 1995b The antitumor sulfonylurea N-(4-methylphenylsulfonyl)-N'-(4-chlorophenyl) urea (LY181984) inhibits NADH oxidase activity of HeLa plasma membranes. Biochimica et biophysica acta **1240:** 11-17.

MORRE, D. M., F. GUO and D. J. MORRE, 2003b An aging-related cell surface NADH oxidase (arNOX) generates superoxide and is inhibited by coenzyme Q. Molecular and cellular biochemistry **254:** 101-109.

MORRE, D. M., G. LENAZ and D. J. MORRE, 2000 Surface oxidase and oxidative stress propagation in aging. The Journal of experimental biology **203:** 1513-1521.

MORRE, D. M., C. MEADOWS, B. HOSTETLER, N. WESTON, D. KERN *et al.*, 2008b Age-related ENOX protein (arNOX) activity correlated with oxidative skin damage in the elderly. BioFactors **34:** 237-244.

MORRE, D. M., C. MEADOWS and D. J. MORRE, 2010 arNOX: generator of reactive oxygen species in the skin and sera of aging individuals subject to external modulation. Rejuvenation research **13:** 162-164.

MORRE, D. M., and D. J. MORRE, 2003b Specificity of coenzyme Q inhibition of an aging-related cell surface NADH oxidase (ECTO-NOX) that generates superoxide. BioFactors **18:** 33-43.

MORRE, D. M., D. J. MORRE, W. REHMUS and D. KERN, 2008c Supplementation with CoQ10 lowers age-related (ar) NOX levels in healthy subjects. BioFactors **32:** 221-230.

NAVARRO, F., J. M. VILLALBA, F. L. CRANE, W. C. MACKELLAR and P. NAVAS, 1995 A phospholipid-dependent NADH-coenzyme Q reductase from liver plasma membrane. Biochemical and biophysical research communications **212:** 138-143.

NAVAS, P., J. M. VILLALBA and R. DE CABO, 2007 The importance of plasma membrane coenzyme Q in aging and stress responses. Mitochondrion **7 Suppl:** S34-40.

NIKLOWITZ, P., T. MENKE, W. ANDLER and J. G. OKUN, 2004 Simultaneous analysis of coenzyme Q10 in plasma, erythrocytes and platelets: comparison of the antioxidant level in blood cells and their environment in healthy children and after oral supplementation in adults. Clinica chimica acta; international journal of clinical chemistry **342:** 219-226.

NUTTER, L. M., E. O. NGO, G. R. FISHER and P. L. GUTIERREZ, 1992 DNA strand scission and free radical production in menadione-treated cells. Correlation with cytotoxicity and role of NADPH quinone acceptor oxidoreductase. The Journal of biological chemistry **267:** 2474-2479.

OHTANI, Y., T. IRIE, K. UEKAMA, K. FUKUNAGA and J. PITHA, 1989 Differential effects of alpha-, beta- and gamma-cyclodextrins on human erythrocytes. European journal of biochemistry / FEBS **186:** 17-22.

OLIVEIRA, B. F., J. A. NOGUEIRA-MACHADO and M. M. CHAVES, 2010 The role of oxidative stress in the aging process. TheScientificWorldJournal **10:** 1121-1128.

PETER, A. D., D. J. MORRE and D. M. MORRE, 2000 A light-responsive and periodic NADH oxidase activity of the cell surface of Tetrahymena and of human buffy coat cells. Antioxidants & redox signaling **2:** 289-300.

PETILLO, D., and H. O. HULTIN, 2008 Ubiquinone-10 as an antioxidant. Journal of Food Biochemistry **32:** 173-181.

POGUE, R., D. M. MORRE and D. J. MORRE, 2000 CHO cell enlargement oscillates with a temperature-compensated period of 24 min. Biochimica et biophysica acta **1498:** 44-51.

PRITCHARD, K. A., JR., L. GROSZEK, D. M. SMALLEY, W. C. SESSA, M. WU *et al.*, 1995 Native low-density lipoprotein increases endothelial cell nitric oxide synthase generation of superoxide anion. Circulation research **77:** 510-518.

PRUDOVA, A., M. ALBIN, Z. BAUMAN, A. LIN, V. VITVITSKY *et al.*, 2007 Testosterone regulation of homocysteine metabolism modulates redox status in human prostate cancer cells. Antioxidants & redox signaling **9:** 1875-1881.

PRUVOT, B., V. LAURENS, F. SALVADORI, E. SOLARY, L. PICHON *et al.*, 2010 Comparative analysis of nonaspanin protein sequences and expression studies in zebrafish. Immunogenetics **62:** 681-699.

RADA, B., C. HABLY, A. MECZNER, C. TIMAR, G. LAKATOS *et al.*, 2008 Role of Nox2 in elimination of microorganisms. Seminars in immunopathology **30:** 237-253.

RADA, B., and T. L. LETO, 2008 Oxidative innate immune defenses by Nox/Duox family NADPH oxidases. Contributions to microbiology **15:** 164-187.

REDDY, V. V., D. KUPFER and E. CASPI, 1977 Mechanism of C-5 double bond introduction in the biosynthesis of cholesterol by rat liver microsomes. The Journal of biological chemistry **252:** 2797-2801.

RENSING, L., U. MEYER-GRAHLE and P. RUOFF, 2001 Biological timing and the clock metaphor: oscillatory and hourglass mechanisms. Chronobiology international **18:** 329-369.

RIZVI, S. I., D. KUMAR, S. CHAKRAVARTI and P. SINGH, 2011 Erythrocyte plasma membrane redox system may determine maximum life span. Medical hypotheses **76:** 547-549.

RIZVI, S. I., K. B. PANDEY, R. JHA and P. K. MAURYA, 2009 Ascorbate recycling by erythrocytes during aging in humans. Rejuvenation Res **12:** 3-6.

RODRIGUEZ-AGUILERA, J. C., G. LOPEZ-LLUCH, C. SANTOS-OCANA, J. M. VILLALBA, C. GOMEZ-DIAZ *et al.*, 2000 Plasma membrane redox system protects cells against oxidative stress. Redox report : communications in free radical research **5:** 148-150.

RUTTER, J., M. REICK, L. C. WU and S. L. MCKNIGHT, 2001 Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors. Science **293:** 510-514.

SARAYMEN, R., E. KILIC, S. YAZAR and M. CETIN, 2003 Influence of sex and age on the activity of antioxidant enzymes of polymorphonuclear leukocytes in healthy subjects. Yonsei medical journal **44:** 9-14.

SCHIBLER, U., 2006 Circadian time keeping: the daily ups and downs of genes, cells, and organisms. Progress in brain research **153:** 271-282.

SCHIMMOLLER, F., E. DIAZ, B. MUHLBAUER and S. R. PFEFFER, 1998 Characterization of a 76 kDa endosomal, multispanning membrane protein that is highly conserved throughout evolution. Gene **216:** 311-318.

SEARGEANT, L. E., and R. A. STINSON, 1979 Inhibition of human alkaline phosphatases by vanadate. The Biochemical journal **181:** 247-250.

SHIMIZU, S., M. NARITA and Y. TSUJIMOTO, 1999 Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature **399:** 483-487.

SHIRABE, K., M. T. LANDI, M. TAKESHITA, G. UZIEL, E. FEDRIZZI *et al.*, 1995 A novel point mutation in a 3' splice site of the NADH-cytochrome b5 reductase gene results in immunologically undetectable enzyme and impaired NADH-dependent ascorbate regeneration in cultured fibroblasts of a patient with type II hereditary methemoglobinemia. American journal of human genetics **57:** 302-310.

SHOSHAN-BARMATZ, V., V. DE PINTO, M. ZWECKSTETTER, Z. RAVIV, N. KEINAN *et al.*, 2010 VDAC, a multi-functional mitochondrial protein regulating cell life and death. Molecular aspects of medicine **31:** 227-285.

SIEGEL, D., D. L. GUSTAFSON, D. L. DEHN, J. Y. HAN, P. BOONCHOONG *et al.*, 2004 NAD(P)H:quinone oxidoreductase 1: role as a superoxide scavenger. Molecular pharmacology **65:** 1238-1247.

SINGER-KRUGER, B., R. FRANK, F. CRAUSAZ and H. RIEZMAN, 1993 Partial purification and characterization of early and late endosomes from yeast. Identification of four novel proteins. The Journal of biological chemistry **268:** 14376-14386.

STRITTMATTER, P., 1965 The reaction sequence in electron transfer in the reduced nicotinamide adenine dinucleotide-cytochrome b5 reductase system. The Journal of biological chemistry **240:** 4481-4487.

STUBBS, M., R. L. VEECH and H. A. KREBS, 1972 Control of the redox state of the nicotinamide-adenine dinucleotide couple in rat liver cytoplasm. The Biochemical journal **126:** 59-65.

SU, D., J. M. MAY, M. J. KOURY and H. ASARD, 2006 Human erythrocyte membranes contain a cytochrome b561 that may be involved in extracellular ascorbate recycling. The Journal of biological chemistry **281:** 39852-39859.

SUN, I. L., E. E. SUN, F. L. CRANE, D. J. MORRE, A. LINDGREN *et al.*, 1992 Requirement for coenzyme Q in plasma membrane electron transport. Proceedings of the National Academy of Sciences of the United States of America **89:** 11126-11130.

TAM, N. N., Y. GAO, Y. K. LEUNG and S. M. HO, 2003 Androgenic regulation of oxidative stress in the rat prostate: involvement of NAD(P)H oxidases and antioxidant defense machinery during prostatic involution and regrowth. The American journal of pathology **163:** 2513-2522.

TANG, X. Y., D. PARISI, B. SPICER, D. M. MORRE and D. J. MORRE, 2013 Molecular cloning and characterization of human age-related NADH oxidase (arNOX) proteins as members of the TM9 superfamily of transmembrane proteins. Advances in Biological Chemistry **3:** 187-197.

TARPEY, M. M., C. R. WHITE, E. SUAREZ, G. RICHARDSON, R. RADI *et al.*, 1999 Chemiluminescent detection of oxidants in vascular tissue. Lucigenin but not coelenterazine enhances superoxide formation. Circulation research **84:** 1203-1211.

TESSIER, S., A. CHAPDELAINE and S. CHEVALIER, 1989 Effect of vanadate on protein phosphorylation and on acid phosphatase activity in the canine prostate. Molecular and cellular endocrinology **64:** 87-94.

THINNES, F. P., 1992 Evidence for extra-mitochondrial localization of the VDAC/porin channel in eucaryotic cells. Journal of bioenergetics and biomembranes **24:** 71-75.

TRIFUNOVIC, A., and N. G. LARSSON, 2008 Mitochondrial dysfunction as a cause of ageing. Journal of internal medicine **263:** 167-178.

VALKO, M., D. LEIBFRITZ, J. MONCOL, M. T. CRONIN, M. MAZUR *et al.*, 2007 Free radicals and antioxidants in normal physiological functions and human disease. The international journal of biochemistry & cell biology **39:** 44-84.

VANDUIJN, M. M., K. TIJSSEN, J. VANSTEVENINCK, P. J. VAN DEN BROEK and J. VAN DER ZEE, 2000 Erythrocytes reduce extracellular ascorbate free radicals using intracellular ascorbate as an electron donor. The Journal of biological chemistry **275:** 27720-27725.

VAQUERO, E. C., M. EDDERKAOUI, S. J. PANDOL, I. GUKOVSKY and A. S. GUKOVSKAYA, 2004 Reactive oxygen species produced by NAD(P)H oxidase inhibit apoptosis in pancreatic cancer cells. The Journal of biological chemistry **279:** 34643-34654.

VILLALBA, J. M., F. NAVARRO, F. CORDOBA, A. SERRANO, A. ARROYO *et al.*, 1995 Coenzyme Q reductase from liver plasma membrane: purification and role in trans-plasma-membrane electron transport. Proceedings of the National Academy of Sciences of the United States of America **92:** 4887-4891.

VILLALBA, J. M., F. NAVARRO, C. GOMEZ-DIAZ, A. ARROYO, R. I. BELLO *et al.*, 1997 Role of cytochrome b5 reductase on the antioxidant function of coenzyme Q in the plasma membrane. Molecular aspects of medicine **18 Suppl:** S7-13.

VILLALBA, J. M., and P. NAVAS, 2000 Plasma membrane redox system in the control of stress-induced apoptosis. Antioxidants & redox signaling **2:** 213-230.

VOEGTLIN, C., J. M. JOHNSON and H. A. DYER, 1924 Quantitative estimation of the reducing power of normal and cancer tissue. Journal of Pharmacology and Experimental Therapeutics **24:** 305-334.

WANG, S., R. POGUE, D. M. MORRE and D. J. MORRE, 2001 NADH oxidase activity (NOX) and enlargement of HeLa cells oscillate with two different temperature-compensated period lengths of 22 and 24 minutes corresponding to different NOX forms. Biochimica et biophysica acta **1539:** 192-204.

WANN, J. G., C. S. LIN, L. C. CHANG, Y. H. HSU, C. T. CHIEN *et al.*, 2006 Enhanced expression of glucose transporter 1 on erythrocyte membrane in hemodialysis patients: the possible role in erythrocyte ascorbate recycling. American journal of kidney diseases : the official journal of the National Kidney Foundation **47:** 1055-1063.

WASSMANN, K., S. WASSMANN and G. NICKENIG, 2005 Progesterone antagonizes the vasoprotective effect of estrogen on antioxidant enzyme expression and function. Circulation research **97:** 1046-1054.

YAGIZ, K., P. W. SNYDER, D. J. MORRE and D. M. MORRE, 2008 Cell size increased in tissues from transgenic mice overexpressing a cell surface growth-related and cancer-specific hydroquinone oxidase, tNOX, with protein disulfide-thiol interchange activity. Journal of cellular biochemistry **105:** 1437-1442.

YANTIRI, F., D. J. MORRE, K. YAGIZ, S. BAROGI, S. WANG *et al.*, 1998 Capsaicin-responsive NADH oxidase activities from urine of cancer patients. Archives of biochemistry and biophysics **358:** 336-342.

YING, W., 2008 NAD+/NADH and NADP+/NADPH in cellular functions and cell death: regulation and biological consequences. Antioxidants & redox signaling **10:** 179-206.

ZIDOVETZKI, R., and I. LEVITAN, 2007 Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies. Biochimica et biophysica acta **1768:** 1311-1324.

# Appendix

## Zusammenfassung

Die spezielle Verwendung von intrazellulären Reduktionsäquivalenten zur Reduktion von Substraten in der extrazellulären Umgebung durch die Einbindung von Plasmamembran assoziierten Enzymen und Elektronenträgern ist praktisch allen eukaryotischen Zellen gemein. Es wird als Transplasmamembran Elektronentransport (PMET) bezeichnet und dient hauptsächlich dem Schutz von Zellen und umgebenden Geweben vor exogenen Schäden, die durch reaktive Sauerstoffspezies und freie Radikale verursacht werden.

Die vorliegende Arbeit hatte zum Ziel, die PMET Aktivität von humanen Erythrozyten unter verschiedenen Gesichtspunkten zu charakterisieren. Das Hauptaugenmerk wurde hierbei auf die Untersuchung von Aktivitätsunterschieden zwischen den Geschlechtern gerichtet, es wurden jedoch auch Analysen gemacht, die die Aktivitätsschwankungen von Erythrozyten unter Bedingungen wie erhöhtem osmolaren Stress, Glukoseüberschuss oder Phosphatasehemmung betrafen. Des Weiteren versuchten wir, age-related NADH Oxidasen (arNOX) - Superoxid generierende Komponenten des PMET Systems, die bereits in menschlichen Körperflüssigkeiten detektiert wurden - in roten Blutkörperchen von älteren Testpersonen durch Immunblottings und Reduktions-Aktivitätsassays, basierend auf Ferricytochrom c und Superoxid Dismutase, zu identifizieren. PMET Aktivitäten wurden spektrophotometrisch über die Verfolgung der extrazellulären Reduktion von Ferricyanid und Ferricytochrom c bestimmt, wobei der Einsatz des Letzteren erstmalig zu diesem Zwecke eingeführt und etabliert wurde und es uns ermöglichte, die reduktiven Aktivitäten präziser zu definieren.

Die geschlechtsspezifischen Analysen ergaben, dass sich die Redoxaktivitäten von männlichen und weiblichen Testpersonen in mehreren Aspekten deutlich unterschieden, ein Hinweis darauf, dass die PMET Aktivität auf eine geschlechtsabhängige Weise reguliert wird. Unsere vorläufigen Beobachtungen betreffend die Zellbehandlungen waren bisher nicht eindeutig einordenbar, konnten

aber insofern Hinweise liefern, als dass hyperosmolarer Stress die PMET Aktivität tendenziell erhöhte, wohingegen der Phosphatasehemmer sie eher erniedrigte. Was arNOX anbelangt, konnten unsere Resultate die Hypothese, wonach diese Oxidase möglicherweise in humanen Erythrozyten zu finden sei, nicht unterstützen.

Zusammenfassend fanden wir heraus, dass das Plasmamembran Redoxsystem in humanen Erythrozyten Schwankungen unterliegt, die durch geschlechtsspezifische Faktoren beeinflusst werden, eine Erkenntnis, die wesentlich von der auf Ferricytochrom c basierenden und bisher unpublizierten Methode, die wir etablierten, profitierte.

# Curriculum vitae

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