



# The role of exercise training on oxidative stress and inflammation in vascular diseases

Erica N. Chirico

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**Erica N Chirico**

**Les effets de l'exercice physique sur le stress oxydant  
et l'inflammation dans les maladies vasculaires.**

**The role of exercise training on oxidative stress and  
inflammation in vascular diseases.**

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*"If we could give every individual the right amount of nourishment and exercise, not too little and not too much, we would have found the safest way to health"*

*~~Hippocrates*

**Les effets de l'exercice physique sur le stress oxydant et l'inflammation dans les maladies vasculaires.**

La drépanocytose (SCD) et l'athérosclérose sont deux maladies très différentes et distinctes qui partagent les mêmes caractéristiques. La drépanocytose est une maladie autosomale récessive appartenant à la classe des hémoglobinopathies causée par la mutation de l'hémoglobine (Hb) A en HbS. En réponse à des stress physiques tels que l'hypoxie, l'acidose, la déshydratation ou l'hyperthermie, HbS devient plus vulnérable à la polymérisation et favorise le processus de falciformation des globules rouges. La répétition des cycles de polymérisation et dépolymérisation de HbS altère la forme saine des érythrocytes et conduit aux manifestations cliniques principales de la drépanocytose: anémie, épisodes vaso-occlusifs aigus et crises hémolytiques. Il est aujourd'hui largement admis que le stress oxydatif et l'inflammation jouent un rôle majeur dans la pathogenèse et les conséquences physiopathologiques de la drépanocytose. L'athérosclérose, quant à elle, est une maladie inflammatoire chronique qui se caractérise par l'accumulation de plaques à l'intérieur des parois vasculaires au niveau de l'endothélium. Le stress oxydatif et la mise en jeu de phénomènes inflammatoires sont impliqués dans l'oxydation des lipides de faible densité (LDL), étape essentielle dans la pathogenèse de cette maladie.

D'autre part, l'activité physique est un mécanisme important de modulation bénéfique du stress oxydatif et de l'inflammation au travers de plusieurs voies d'adaptation : l'amélioration des enzymes antioxydantes, de la vasodilatation et des cytokines anti-inflammatoires, et la baisse des contraintes de cisaillement. Nous avons donc cherché à déterminer dans ce travail

de thèse comment le contrôle du stress oxydatif et de l'inflammation par l'activité physique pourrait réduire les complications de ces 2 pathologies (SCD et athérosclérose).

Dans le chapitre d'introduction de cette thèse nous avons présenté les 2 pathologies étudiées dans ce travail (drépanocytose, trait drépanocytaire et athérosclérose) puis, nous avons discuté les effets de l'exercice aigu et chronique sur le stress oxydatif et l'inflammation.

Le deuxième chapitre est constitué tout d'abord d'une revue de littérature sur les causes et conséquences du stress oxydatif dans la drépanocytose, qui a été publiée dans IUBMB Life. Cette section discute ensuite l'état actuel des connaissances des effets de l'exercice physique aigu et chronique sur la physiopathologie de la drépanocytose et du trait drépanocytaire. La synthèse de ce 2<sup>e</sup> chapitre justifie la première étude expérimentale de cette thèse publiée dans le Journal of Applied Physiology : "Exercise training blunts oxidative stress in sickle cell trait carriers". Dans cette étude, nous avons observé que i) les porteurs du trait drépanocytaires (HbAS) avaient un stress oxydatif plasmatique exacerbé en réponse à un exercice physique intense et ii) que l'activité physique régulière réduisait ce stress oxydatif à des niveaux similaires aux sujets en bonne santé. Cette diminution du stress oxydatif semblait s'expliquer par l'amélioration des antioxydants. Cette réponse bénéfique sur le stress oxydatif était aussi corrélée à une diminution de l'adhérence vasculaire. Parce que le stress oxydatif et l'adhésion vasculaire jouent un rôle central dans la pathophysiologie de la drépanocytose, il est raisonnable de penser que l'entraînement physique pourrait aussi induire des adaptations bénéfiques similaires chez les patients drépanocytaires (HbSS).

Le 3<sup>e</sup> chapitre commence avec une synthèse bibliographique des effets de l'activité physique sur l'inflammation et le stress oxydatif dans la pathogénèse de l'athérosclérose. Cette revue est en cours de mise en forme pour être soumise dans Exercise & Sport Sciences Reviews ("The

role of exercise training on the effects of inflammation and oxidative stress in the pathogenesis of atherosclerosis”). La section suivante aborde succinctement les techniques d'imagerie et de l'utilisation de modèles animaux d'athérosclérose. La fin ce chapitre est conclu par la problématique de la deuxième étude de cette thèse: "MRI evidence of exercise-induced improvement of oxidative stress and inflammation in old ApoE<sup>-/-</sup> mice" qui est soumise dans Circulation. Cette étude a déterminé les effets de la course volontaire chez la souris ApoE<sup>-/-</sup> âgées sous un régime high-fat/high-cholesterol. Grâce à l'IRM, nous avons montré que l'entraînement physique réduisait la superficie de la paroi de l'aorte et changeait la composition des plaques aortiques. En outre, dans le cerveau, l'entraînement physique réduisait l'infiltration des macrophages mesuré par IRM et immunohistologie, le stress oxydatif et l'inflammation. Ces résultats ont été confirmés par des marqueurs plasmatiques du stress oxydatif et de l'inflammation. Au final, cette étude nous a permis de démontrer que l'entraînement physique pouvait être bénéfique dans la réduction des complications de l'athérosclérose même à un stade avancée.

En conclusion, ce travail de thèse démontre que l'entraînement physique peut modifier le stress oxydatif et l'inflammation dans deux maladies vasculaires distinctes. D'une part, l'entraînement physique permet aux sujets porteurs du trait drépanocytaires de revenir à des valeurs plasmiques similaires à des sujets sains en réponses à un exercice physique maximal. D'autre part dans notre modèle d'athérosclérose, nous avons montré que l'entraînement physique peut exercer une réponse bénéfique en limitant l'accumulation des macrophages dans le cerveau ainsi que dans l'aorte.

En conclusion générale, l'ensemble de nos résultats démontrent la puissance de l'entraînement physique pour modifier un état pathologique déséquilibré vers une situation plus homéostatique. Les analyses mécanistiques sont en cours pour une meilleure compréhension

de la modification du trafic des macrophages et de leur polarisation avec l'entraînement d'endurance.

Les mots clés: drépanocytose, athérosclérose, entraînement physique, stress oxydatif, inflammation, adhésion vasculaire

## English Summary

The role of exercise training on oxidative stress and inflammation in vascular disease.

Sickle cell disease (SCD) and atherosclerosis are two very different and distinct diseases that share similar underlying characteristics. Sickle cell disease is a hemoglobinopathy characterized by a genetic mutation which causes the normal blood cells to become rigid and weak. The resulting pathophysiological effects, including sickling, vaso-occlusion, and adhesion, involve the production of oxidative stress and inflammation. Atherosclerosis is a chronic inflammatory disease that is characterized by plaque buildup within the vessel walls. An initial step in the pathogenesis of this disease involves the oxidation of lipids, which not only produces inflammation, but more oxidative stress as well. We sought to determine how the control of oxidative stress and inflammation could ameliorate complications stemming from the disease.

Exercise training is an important mechanism for the beneficial modulation oxidative stress and inflammation through several adaptive pathways: antioxidants, shear stress, vasodilation, and anti-inflammatory cytokines. The purpose of this thesis was to determine if these beneficial effects of exercise training could improve oxidative stress and consequently inflammation in sickle cell trait (SCT) and atherosclerosis.

The introduction chapter of this thesis will discuss general background information on sickle cell disease/trait and atherosclerosis, followed by a brief discussion on the role of oxidative stress and inflammation in exercise.

The second chapter will begin with my review published in IUBMB Life on oxidative stress in sickle cell disease. This section will discuss the current knowledge on the effects of

exercise in sickle cell disease and sickle cell trait. Knowledge obtained from this chapter will provide evidence for the importance of the first discussed study published in the Journal of Applied Physiology: “Exercise training blunts oxidative stress in sickle cell trait carriers”. In this study, we found that SCT carriers experience more oxidative stress and have an impaired production of anti-oxidants. Habitual physical activity was able to reduce oxidative stress and improve anti-oxidants in response to an acute exercise bout. This response was related to an improvement of cell adhesion. We demonstrated that exercise training allows SCT carriers to respond to acute stresses in a manner similar to control healthy subjects. Because these factors (oxidative stress, vascular adhesion) play a central role in SCD, exercise training could also elicit similar responses in SCD patients.

The next chapter will begin with another review (to be submitted), “The role of exercise training on the effects of inflammation and oxidative stress in the pathogenesis of atherosclerosis”. The following section will briefly cover imaging techniques and the use of animal models of atherosclerosis. The information thus far will provide the motivation for developing my second study, “Exercise training reverses the age-associated changes in atherosclerosis: the role of inflammation and oxidative stress”. This study looked at the effects of voluntary wheel running on aging ApoE<sup>-/-</sup> mice on a high-fat/high-cholesterol diet. Using MRI, we found that exercise training reduced the vessel wall area of the aorta and changed aortic plaque composition. In the brain, exercise training reduced macrophage infiltration, oxidative stress, and inflammation. These results were confirmed by plasma markers of oxidative stress and inflammation. We demonstrated that exercise training can be beneficial in reducing the complications in advanced atherosclerosis.

In conclusion, we found that exercise training can modify the production of oxidative stress and inflammation in two distinct diseases. In SCT, exercise training allows carriers to respond

to a stressful stimulus as a healthy control. In atherosclerosis, we found that exercise training can exert a beneficial response to macrophage accumulation in the brain as well as the aorta. Taken together, these results demonstrate the power of exercise training to alter an unbalanced disease state towards a more homeostatic situation. Perspectives in mechanistic analysis are ongoing for a better understanding of the modification of macrophage trafficking and polarization with endurance training.

Key words: sickle cell disease, atherosclerosis, exercise training, oxidative stress, inflammation, adhesion



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## Abbreviation List

**·OH:** Hydroxyl radical

**ACS:** acute chest syndrome

**A-HOO:** ascorbate

**A-HOOH:** ascorbic acid; vitamin C

**A-OO:** dehydroascorbate

**ApoE:** apolipoprotein E

**BBB:** Blood brain barrier

**BH<sub>4</sub>:** tetrahydrobiopterin

**eNOS:** endothelial nitric oxide synthase

**e-selectin:** endothelial selectin

**ETC:** electron transport chain

**ExT:** exercise trained

**GPX:** glutathione peroxidase

**GSH:** reduced glutathione

**GSSG:** glutathione disulfide

**H<sub>2</sub>O<sub>2</sub>:** hydrogen peroxide

**HbA:** normal hemoglobin

**HbS:** heterozygous sickle hemoglobin (sickle cell trait)

**HbSS:** homozygous sickle hemoglobin (sickle cell disease)

**HDL:** high density lipoprotein

**HO-1:** heme oxygenase-1

**HU:** Hydroxyurea

**ICAM-1:** intracellular adhesion molecule-1

**IFN:** interferon

**IL:** interleukin

**iNOS:** inducible nitric oxide synthase

**LDH:** lactate dehydrogenase

**LDL:** low density lipoprotein

**l-selectin:** leukocyte selectin

**M1:** classically activated macrophages (pro-inflammatory)

**M2:** alternatively activated macrophages (anti-inflammatory)

**MAP:** mean aerobic power

**MCP-1:** monocyte chemoattractant protein-1

**MPO:** myeloperoxidase

**MRI:** magnetic resonance imaging

**NAC:** N-acetylcysteine

**NADPH:** nicotinamide adenine dinucleotide phosphate

**NF- $\kappa$ B:** Nuclear Factor- $\kappa$ B

**NO:** nitric oxide

**NO<sub>x</sub>:** nitric oxide metabolites

**O<sub>2</sub><sup>•-</sup>:** superoxide

**ONOO<sup>-</sup>:** peroxynitrite

**oxLDL:** oxidized low density lipoprotein

**PHT:** pulmonary hypertension

**p-selectin:** platelet selectin

**qPCR:** quantitative polymerized chain reaction

**RBC:** red blood cell

**ROS:** reactive oxygen species

**SCD:** sickle cell disease

**SCT:** sickle cell trait

**SOD:** superoxide dismutase

**T1:** longitudinal relaxation time

**T2\*:** transversal relaxation time in inhomogenous fields

**T2:** transversal relaxation time

**TBARS:** thiobarbituric acid reactive substances

**TCD:** transcranial doppler

**TNF:** tumor necrosis factor-alpha

**TRV:** tricuspid regurgitant jet velocity

**USPIO:** ultrasmall superparamagnetic iron oxide particles

**UT:** untrained

**VCAM-1:** vascular adhesion molecule-1

**VO<sub>2</sub>:** oxygen consumption

**VOC:** vaso-occlusive crisis

**XO:** xanthine oxidase

# Chapter I

## General Introduction



## Chapter I. General Introduction.

### I.1 Sickle Cell Disease vs. Atherosclerosis.

Sickle cell disease (SCD) and atherosclerosis are two very different vascular diseases that share similar underlying characteristics.

- Sickle cell disease is a hemoglobinopathy characterized by a genetic mutation which causes the normal blood cells to become rigid and weak. The resulting pathophysiological effects, including sickling, vaso-occlusion, and adhesion, involve the production of oxidative stress and inflammation.
- Atherosclerosis is a chronic inflammatory disease that is characterized by plaque buildup within the vessel walls. An initial step in the pathogenesis of this disease involves the oxidation of lipids, which not only produces inflammation, but more oxidative stress as well.

Although both sickle cell disease and atherosclerosis have clinically different pathologies, similar patterns occur in these conditions. Briefly described above (and described more fully in Chapters III and IV), SCD and atherosclerosis involve oxidative stress and inflammation. As illustrated in Figure 1, an increase in oxidative stress occurs either from an increase in reactive oxygen species (ROS) or a decrease in nitric oxide (NO) bioavailability. ROS can stimulate the production of inflammatory cytokines and adhesion molecules, while a decrease in nitric oxide can also influence adhesion as well as vaso-constriction. Producing a cyclic condition, a chronic inflammatory and oxidative state ensues, resulting in a cardiovascular response. Depends on the disease, the response can differ. In sickle cell disease conditions

such as acute chest syndrome, vaso-occlusive crises, and pulmonary hypertension can occur, while in atherosclerosis some include myocardial infarction and cerebrovascular disease.

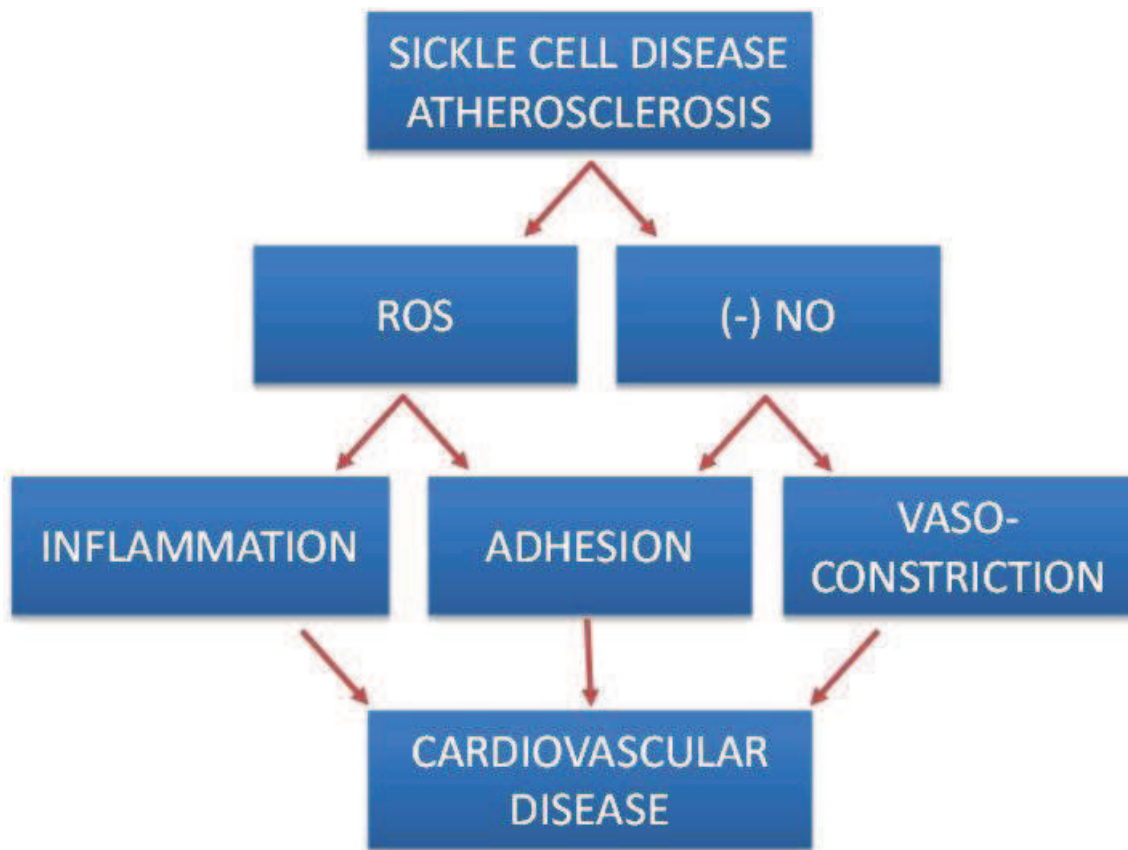


Figure 1: Overlapping pathways of SCD and atherosclerosis. Both conditions involve an increase in oxidative stress as characterized by an increase of ROS and an impairment of nitric oxide. The combination of these two lead to a cyclic reaction of increased inflammation, adhesion, and vasoconstriction, resulting in cardiovascular complications.

## I.2 Exercise Training.

We chose exercise training as an intervention because it is a non-invasive and simple method that can be implemented anywhere by anyone. Physical inactivity and a sedentary lifestyle are independent risk factors for a plethora of diseases and disorders. On the other hand, exercise

training prevents the development of vascular disease and reduces cardiovascular mortality. However, exercise has historically been a method of survival and it should not be considered an additional “treatment”, but rather as a method to revert our systems to a natural homeostasis.

Exercise training is an important mechanism for the beneficial modulation of oxidative stress and inflammation through several adaptive pathways: antioxidants, shear stress, vaso-dilation, and anti-inflammatory cytokines. As sickle cell disease and atherosclerosis are overwhelmed by these factors, we believe that exercise training could be a useful method for controlling and understanding the underlying mechanisms.

**Chapter II:**

**Oxidative Stress**

**&**

**Inflammation**

## Chapter II: Oxidative Stress and Inflammation

### II.1 Oxidative Stress

The term oxidative stress refers essentially to the damaging imbalance between the production of oxidants, such as reactive oxygen species (ROS), and the production of anti-oxidants (1). Excess ROS that are not neutralized by the body's defense system can damage vital cellular components causing abnormal gene expression, cell proliferation, or cell death. These imbalances can be a result of normal metabolic processes, aging, a large number of disease states (1), high intensity or acute exercise (2), or hypoxic conditions (3). However, ROS are not always a negative component in the body. ROS play a vital role in gene activation and cellular growth, and function in the immune system as a defense against bacteria and viruses. ROS are also responsible for the dilation of blood vessels; they participate in blood pressure control and they mediate the biosynthesis of prostaglandins. ROS also have a critical role in cellular signaling; participating as second messenger systems within redox regulation [See reviews (4, 5)].

Molecular oxygen is essential for the proper functioning of all aerobic organisms. Oxygen itself is considered an oxidant due to its electron accepting capabilities, functioning as a strong oxidizing agent (1, 2). During its four electron reduction to water, highly reactive metabolites are formed, including superoxide anion radical ( $O_2^{\cdot -}$ ), hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radical (OH). These reduced metabolites of oxygen are referred to as "reactive oxygen species" (1) (See Figure 2). ROS can induce oxidative damage to the cell due to its tenacity to form a very stable structure by extracting electrons from other sources (2).

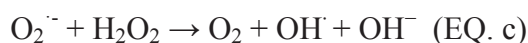
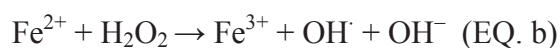
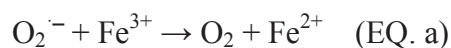
In order to protect the body against the possible damaging effects of ROS, anti-oxidant enzymes are produced in the cells (1). A common definition by Halliwell & Gutteridge states that an antioxidant is a “substance, that when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate” (6). These protective mechanisms include the enzymatic anti-oxidants; superoxide dismutase, catalase, glutathione peroxidase, and nitric oxide, and the non-enzymatic anti-oxidants; tocopherols, glutathione, carotenoids, ascorbic acid, and uric acid. These low-molecular weight anti-oxidants, can directly and indirectly affect ROS. They have a direct effect through electron donation, in which the radical is scavenged, thereby preventing it from attacking a biological target (4). They can also indirectly prevent ROS formation through the chelating of transition metals, which could participate in the iron-catalyzed Haber-Weiss reaction (7) (See Figure 2).

Under normal conditions, the generation of ROS is combated by the production of anti-oxidants resulting in harmless byproducts. Superoxide is formed from the one electron reduction of oxygen during catalytic events or leaks in electron transfer reactions. It can be rapidly dismutated into hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and oxygen spontaneously or via superoxide dismutase (SOD), a metalloproteinase which provides a defense against cellular damage.



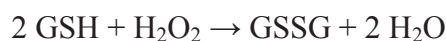
$\text{H}_2\text{O}_2$  is not a radical itself although it is still classified as a ROS due to its damaging affects at certain concentrations and its ability to form hydroxyl radical ( $\text{OH}^{\cdot}$ ). The presence of  $\text{H}_2\text{O}_2$  has the ability to increase the generation of ROS 8-fold (8). Due to the combination of the Fenton and the Haber-Weiss reactions,  $\text{OH}^{\cdot}$  can be produced *in vivo* (4). The Haber-Weiss

reaction reduces ferric ions to ferrous ions via superoxide (EQ. a). The reduced ferrous ions can now react with  $\text{H}_2\text{O}_2$  to form  $\text{OH}^\cdot$  via the Fenton reaction (EQ. b). The sum of these two reactions, leads to the slow but feasible production of  $\text{OH}^\cdot$  *in vivo* (EQ. c).

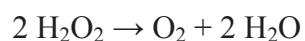


Hydroxyl radical is a highly damaging and powerful oxidizing agent; considered the most reactive in the biological system (4).

To combat this cyclic production of  $\text{OH}^\cdot$ ,  $\text{H}_2\text{O}_2$  can be reduced before it has a chance to be converted to  $\text{OH}^\cdot$ . The disposal of  $\text{H}_2\text{O}_2$  relies equally on the actions of glutathione peroxidase (GPX) and catalase, two powerful anti-oxidants. GPX has a high affinity for  $\text{H}_2\text{O}_2$  and can remove it in low concentrations, resulting in water and oxidized glutathione (GSSG). It is however a metabolically expensive process; requiring 2 molecules of reduced glutathione (GSH) for 1 molecule of  $\text{H}_2\text{O}_2$  (4).



Catalase, a heme-containing enzyme (7), has a high  $K_m$  (Michaelis constant; indicates the substrate concentration at 1/2 the maximum velocity) and can remove  $\text{H}_2\text{O}_2$  at high concentrations (4), forming water and oxygen.



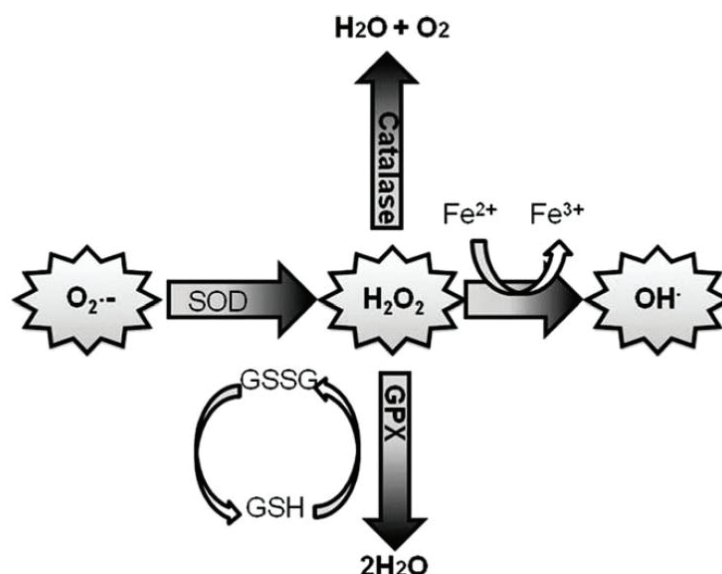


Figure 2: Balance of ROS and antioxidants. Oxidative stress is the imbalance between the production of ROS and antioxidants. The antioxidant properties of GPX, SOD, and catalase control the production of oxygen species.

Xanthine oxidase (XO) is an enzyme which under hypoxic conditions results in the generation of superoxide. It oxidizes hypoxanthine to xanthine and further oxidizes xanthine to uric acid; during each step of the conversions  $2 \text{H}_2\text{O}_2$  and  $2 \text{O}_2^{\cdot-}$  are formed.

The cellular waste product, uric acid, is an effective endogenous anti-oxidant. Uric acid in its physiological state can react with hydroxyl radical forming a stable radical. It can also chelate metals ions (copper and iron) and prohibit their interaction in the redox cycle (4).

Glutathione, or GSH in its reduced form and GSSG in its oxidized form, is an abundant and critical anti-oxidant that can limit oxidative stress via the direct scavenging of reactive oxygen species, such as  $\text{OH}^{\cdot}$  (9). It is also a cofactor for the reactions involving the decomposition of  $\text{H}_2\text{O}_2$  by the enzyme peroxidase, and can indirectly chelate copper ions (4).



Ascorbic acid (vitamin C, A-HOOH) is an important and powerful scavenger and reducing agent that can donate 2 electrons to oxygen species such as hydroxyl, peroxy, and thyl radicals, thereby eliminating them. Ascorbate (A- HOO) and dehydroascorbate, (A- OO) are respectively formed from the 1- and 2- electron oxidation of ascorbic acid.



Ascorbate and dehydroascorbate can be regenerated into ascorbic acid through the obtention of 1 and 2 electrons, respectively, from GSH. Ascorbic acid can also react with molecular oxygen to regenerate other anti-oxidants such as reduced tocopherol. Tocopherols (vitamin E) have the ability to provide stability to cellular membranes, as well as scavenge sources of ROS, such as molecular oxygen.

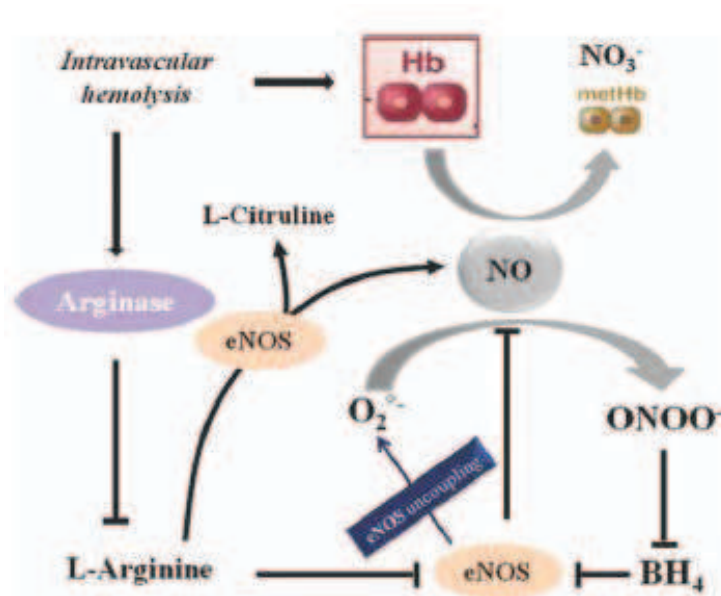
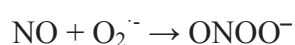


Figure 3: Degradation of NO. NO is decreased in three ways: through the reaction with O<sub>2</sub><sup>·-</sup> forming ONOO<sup>-</sup> and through its inhibition via the byproducts of hemolysis.

Nitric oxide (NO) is a vital enzyme which controls the rate of relaxation in blood vessels via both its cyto-protective and cyto-toxic characteristics. It is a diffusible intracellular messenger produced by most mammalian cells including vascular endothelial cell, smooth muscle cells, macrophages, and platelets. Three different isoforms can generate NO enzymatically by NO synthase (NOS): neuronal NOS (nNOS or NOS1); inducible NOS (iNOS or NOS2); and endothelial or constitutive NOS (eNOS or NOS3).

For the purpose of this thesis, only eNOS will be discussed in this section as it relates more significantly to oxidative stress. iNOS will be discussed in the section on inflammation as it is induced by inflammatory cytokines.

eNO is a major endothelial-derived relaxation factor that is important to the cardiovascular and peripheral vascular systems. Formed by endothelial nitric oxide synthase (eNOS), it diffuses to the adjacent smooth muscle cells where a reaction with ferrous iron of the guanylate cyclase heme group causes synthesis of cyclic guanosine monophosphate (10). It is responsible for smooth muscle cell relaxation and vasodilation; controlling 25% of resting blood flow (10). Through its regulation of vascular homeostasis and its control of basal and stimulated vasomotor tone, it plays a major role in inhibiting the actions of reperfusion injury and endothelial proliferation. NO's cyto-protection can be made negative when the concentration of superoxide exceeds that of NO. As the concentration increases, NO binds to superoxide which inhibits the vasodilatory effects of NO and instead produces the oxidant peroxynitrite (ONOO<sup>-</sup>) (7). See Figure 3.





## II.2 Inflammation

Inflammation is a protective biological response initiated by the immune system that involves complex communication between immune cells to respond to harmful stimuli such as pathogens, damaged cells, or irritants. Acute inflammation consists of leukocyte recruitment from the circulation, with initial recruitment of polymorphonuclear granulocytes followed by monocytes and macrophages. However, chronic inflammation, as found in cardiovascular disease, can lead to destruction of tissues. The inflammatory response involves the regulation of pro- and anti-inflammatory mediators in resident tissue cells and recruited leukocytes through the coordination of various signaling pathways.

Cytokines are mediators of the inflammatory response and are classified into several classes: interleukins; tumor necrosis factors (TNF); interferons (IFN), colony stimulating factors (CSF), transforming growth factors (TGF), and chemokines. Helper-T (Th) cells are principal players in adaptive immunity and are classified according to the cytokines they secrete. A cell-mediated immune response (Th1) is associated with the secretion of IL-2 and IFN- $\gamma$ , while a humoral immune response (Th2) is associated with the secretion of IL-4, IL-5, IL-10, and IL-13. Cytokines are also characterized based on the pathway in which they mediate their effects. Most ILs, CSFs, and IFNs belong to the JAK-STAT pathway. IL-1 (including IL-1 $\alpha$ , IL-1 $\beta$ , IL1ra, and IL-18) and TNF activate NF- $\kappa$ B and MAP kinase signaling pathways, while TGF- $\beta$  superfamily members activate signaling proteins of Smad family (See Figure 4). Cytokines are classified according to either pro- (IL1/12/18, IFN- $\gamma$ , TNF) or anti- (IL4/10/13, TGF-B) inflammatory activities.

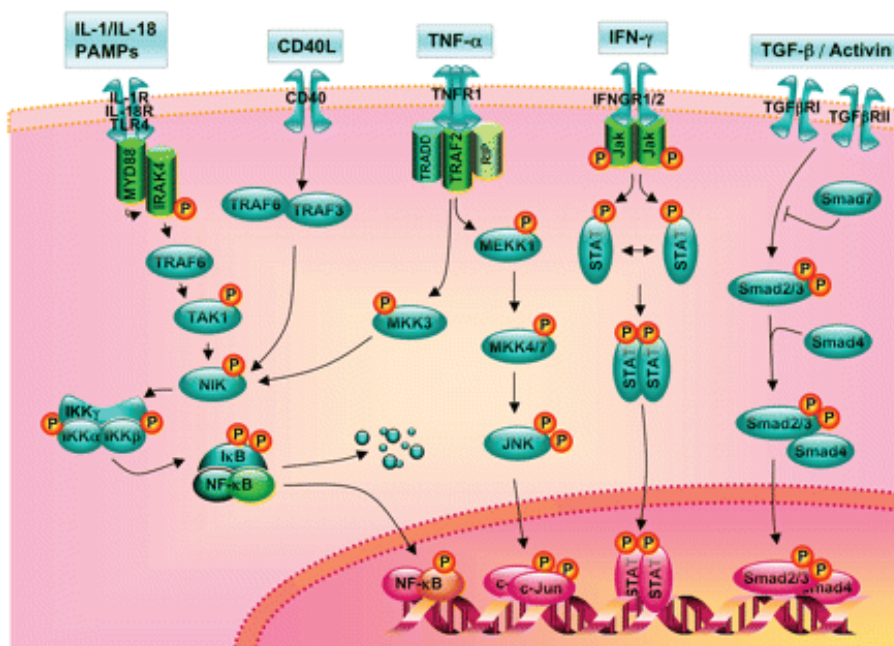


Figure 4: Pathway of the various signaling cascades. NF-κB and MAP kinase signaling pathways are activated by interleukins (IL), colony stimulating factor (TGF) and interferons (IFN). SMAD family are activated by transforming growth factors (TGF) (11).

## II.2a Cytokine Signaling Pathways

**NF-κB.** NF-κB is one of the principle pathways activated in response to pro-inflammatory cytokines such as TNF-α, IL1, and IL-18. In its inactive form, NF-κB is sequestered in the cytoplasm by inhibitor proteins, IκBs. Various stimuli that activate NF-κB cause the subsequent degradation of IκB. The activated NF-κB complex can then translocate into the nucleus and induce gene expression. This negative-feedback loop gives rise to oscillations in NF-κB translocation. Its activation regulates genes encoding pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes such as COX2 and iNOS.

NF-κB is a redox-sensitive transcription factor and is highly influenced by the intracellular redox status of the cell (11). Antioxidants, such as aspirin, NAC, and flavonoids, can inhibit

activation of NF- $\kappa$ B. HO-1 is an example of an anti-inflammatory pathway induced in response to TNF and IL-1-induced inflammation (12, 13).

*JNK/AP-1.* JNK phosphorylation is mediated by two MAPK kinases: MAP2K4 and MAP2K7. JNK pathway regulates many pro-inflammatory genes, including those encoding for TNF- $\alpha$ , IL-2, IL-6, E-selectin, ICAM-1, VCAM-1, MCP-1, COX2, and MMPs (-1, -9, -12, -13).

*JAK/STAT.* JAK and/or STAT proteins can be activated by IFNs, as well as several cytokines (especially IL-6), growth factors, and hormonal factors. IL-4 activates STAT6 and promotes differentiation of Th2 cells (14), while IL-12 activates STAT4 and promotes differentiation of IFN $\gamma$ -producing Th1 cells (15).

*SMADS.* TGF- $\beta$ -triggered signals are transduced by Smad family proteins. Smad3 has antagonistic properties and plays a major role in TGF- $\beta$ -dependent repression of vascular inflammation by inhibiting AP-1 activity (16). IFN $\gamma$ -induced expression of Smad7 acts as a negative regulator of the TGF- $\beta$ /Smad pathway (17).

For example, in atherosclerosis plaque progression and resolution is regulated by tightly controlled and opposing cytokine pathways. See Figure 5

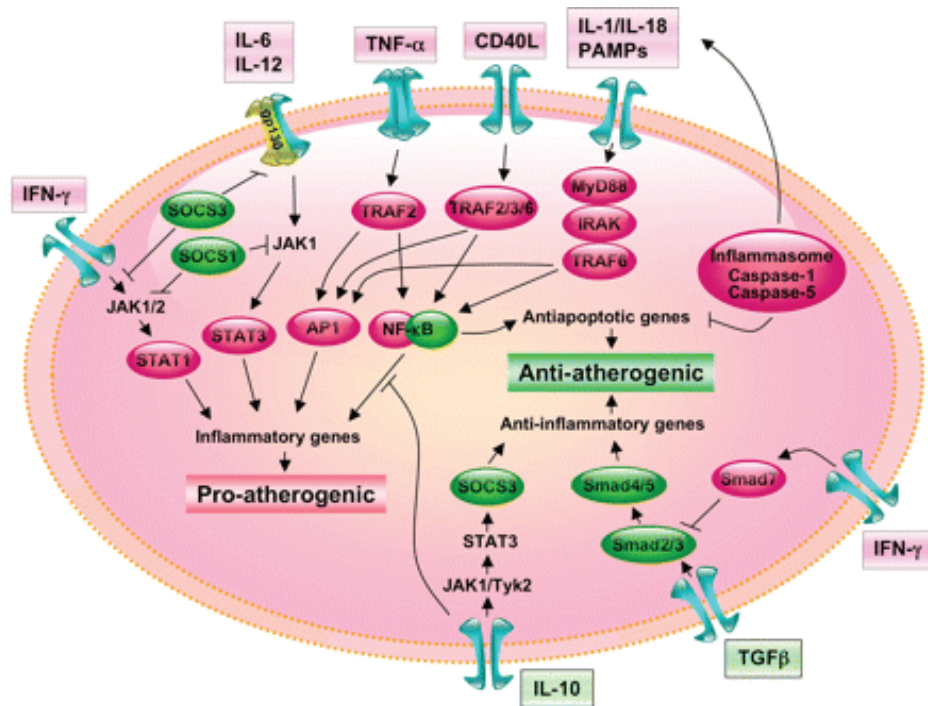


Figure 5: Opposing signaling pathways. Pro-inflammatory cytokines leading to a pro-atherogenic response include IFN- $\gamma$ , TNF $\alpha$ , and interleukins. Anti-inflammatory cytokines leading to an anti-atherogenic response include IL-10 and TGF $\beta$  (11).

### II.3 Link between Oxidative Stress & Inflammation

ROS such as  $O_2^{\cdot -}$ ,  $H_2O_2$ , and  $OH^{\cdot}$  are produced in response to activation by cytokines, including TNF $\alpha$  and IL-1. The production of ROS activates redox-sensitive signaling pathways that induce inflammatory gene expression. In adequate quantities, ROS are considered to be second messengers. ROS have been considered to be general messengers for the induction of NF- $\kappa$ B activation (19), although possibly not through direct mechanisms (20). Recent evidence supports the notion the ROS may oxidize NF- $\kappa$ B subunits, thereby impairing DNA binding and transcriptional activities of NF- $\kappa$ B (21). In addition, ROS can strongly activate JNK through the oxidative inactivation of the endogenous JNK inhibitors (21).

iNOS is produced as a result of endotoxin and cytokine activation. It produces large, toxic amounts of NO in a sustained manner to help kill or inhibit the further growth of invading microorganisms. Cytokines released from infected host cells, such as TNF- $\alpha$  and IL-1 $\beta$ , can activate NO production. The NF $\kappa$ B pathway and IFN- $\gamma$ , via the activation of JAK and STAT pathways, can trigger iNOS transcription (22, 23). While iNOS is protective against certain infectious diseases chronic inflammatory conditions can cause DNA damage or tumorigenesis (24). Anti-inflammatory cytokines such as TGF- $\beta$ , IL-4, or IL-10 (25), and PPAR $\gamma$  (26) can inhibit these pathways, reducing iNOS production.



## II.4 Effects of Exercise on Oxidative Stress & Inflammation

Physical inactivity accentuates the negative components of diseases such as atherosclerosis, diabetes, obesity, and metabolic syndrome (27). On the other hand, exercise training is able to reduce and prevent these diseases (28, 29). It is now well documented that acute exercise increases ROS and inflammation. However, the body adapts to a continual practice of exercise by increasing anti-oxidants and anti-inflammatory agents to compensate for the overproduction of ROS and inflammation. In this section, I will review the role of acute and chronic exercise on oxidative stress and inflammation.

### II.4a Acute Exercise.

#### *Oxidative Stress.*

Aerobic exercise can cause an increase of up to 15-fold in the rate of oxygen consumption throughout the body, and as much as 100-fold in the oxygen flux in active muscles (30). As oxygen consumption and flow increases, so does the possibility of ROS production. There are several theories as to how ROS is produced during exercise, including i) leakage in mitochondrial electron transport chain, ii) ischemia/reperfusion, and iii) neutrophils.

It is commonly understood that inadequate coupling of electron transfers between complexes in the mitochondrial electron transport chain (ETC) causes the leak of superoxide radicals (31, 32). However, other studies have suggested that a decrease in mitochondrial  $pO_2$  is the cause of exercise-induced increase in ROS production rather than an ETC leakage (32, 33). This is supported by studies that show that isometric exercises do not induce an increase in  $VO_2$  but still causes an increase in oxidative stress possibly due to reduce mitochondrial  $pO_2$  (34).

Secondly, another hypothesis suggests that mechanisms similar to ischemia-reperfusion injury could be responsible for ROS increase. During exercise blood flow is redistributed to active tissues leading to transient tissue hypoxia. The ischemic condition could trigger the production of XO. The reoxygenation period that follows could produce superoxide from XO. Studies have found an increase in superoxide and XO levels following exercise (35, 36), which can be ameliorated using allopurinol, an XO inhibitor (36–38).

Finally, cytokines released during an acute bout of exercise can facilitate an influx of leukocytes at the site of inflammation. Neutrophils can infiltrate affected areas, leading to a respiratory burst which produces ROS such as superoxide and hydrogen peroxide. Within neutrophils is an iron-containing enzyme called myeloperoxidase (MPO) that can convert hydrogen peroxide into hypochlorous acid, a highly potent oxidant. It has been demonstrated that exercise leads to an increase in neutrophil and MPO levels (40–43).

The consensus of most human and animal studies has found that acute aerobic (44–46) and anaerobic (46, 47) exercise increases oxidative stress. In 1978, Dillard et al (49) found that exercise at 75%  $V_{O2max}$  increased levels of lipid peroxidation compared to resting subjects. It was then shown that exhaustive exercise increased liver and muscle free radical concentration two- to three-fold (44). Long duration exercise also increases free radical concentration in skeletal muscle and myocardium (50). However, other studies show no change in acute exercise compared to control. The discrepancies between results can be due to intensity (48, 49) and duration (50) of the exercise protocol, training status of subjects (51–53), and age (54).

**Antioxidants.** In response to acute physical activity, the body's antioxidant defense system may be temporarily reduced as it combats the increase in ROS. Therefore, the measurement of

antioxidants can be used as a marker of oxidative stress. During exercise and immediately after the exercise bout, antioxidants may be reduced (55, 56) indicating an increase in ROS. Further into the recovery period, antioxidants may increase above basal levels (34, 55–57) indicating a return to more normal levels of ROS. It is possible that those studies which found no significant changes in anti-oxidant capacity may not have taken enough samples throughout the protocol (44, 58).

### ***Inflammation.***

**Pro-inflammation.** During acute exercise, active muscles secrete several cytokines into the circulation. Strenuous exercise can induce an increase in TNF $\alpha$ , followed by IL-1 $\beta$ , and then IL-6. Most studies have found that strenuous exercise increases levels of IL-6 up to 100-fold while TNF $\alpha$  can increase 2-3 fold. Although some studies have found that exercise can increase IL-1 (59–61) and TNF $\alpha$  (61, 62), other studies have found no detectable response in IL-1 (63) or TNF $\alpha$  (67, 68). Most of these studies have looked at the response after a marathon; a long and strenuous exercise bout which may produce an excess of cytokines.

Most studies consistently agree that IL-6 is increased in response to exercise. IL-6 is released from contracting skeletal muscle after 30 minutes of exercise and up to 100-fold increases in IL-6 mRNA may be present by the end of exercise (69, 70). The amount of IL-6 increase is related to the exercise intensity (68) and quantity of muscle used in the exercise (69) which is why most studies have observed the greatest increase in running experiments (i.e. the marathon) (68, 70, 71).

**Anti-Inflammation.** IL-6 is usually referred to as an “inflammation-responsive” cytokine as it does not directly induce inflammation. It has been shown that IL-6 can stimulate the

production of anti-inflammatory agents, as it is associated with increased IL-1 $\alpha$ , IL-10, and TNF- $\alpha$  receptors (75, 76) (see Figure 6), while exerting an inhibitory effect on TNF- $\alpha$  and IL-1 production (74). This was demonstrated in a mouse model of IL-6 deficiency, as exercise only modestly increased levels of TNF- $\alpha$  (75)

In addition to IL-6, IL-10 and IL-1ra also appear in the circulation following exercise. IL-10 has been suggested to inhibit the production of IL-1 $\alpha$ , IL-1b, TNF- $\alpha$ , IL-8, and macrophage inflammatory protein- $\alpha$  (MIP- $\alpha$ ) (76, 77). The main role of IL-1 $\alpha$  is to inhibit signaling transduction through the IL-1 receptor complex, inhibiting the actions of the IL-1 family (78).

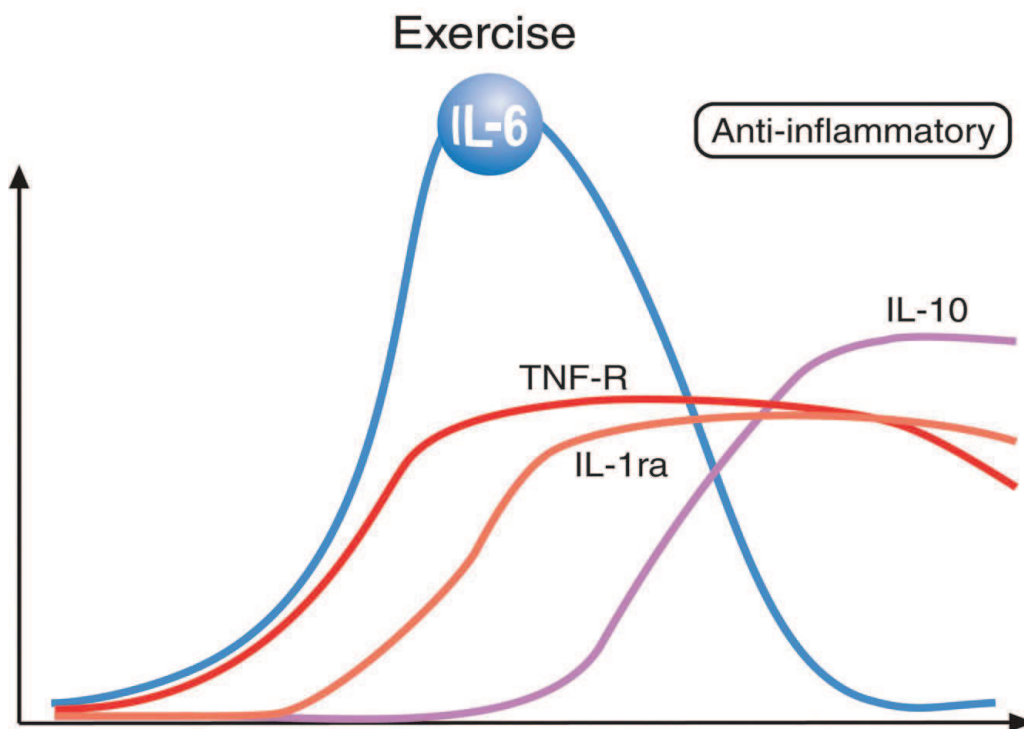


Figure 6: The anti-inflammatory response to acute exercise. Pro-inflammatory cytokine IL-6 can stimulate an anti-inflammatory response; inducing TNF-R, IL-1ra, and IL-10 at different time points throughout an exercise bout (79).

#### II.4b Chronic Exercise.

Chronic exercise is a habitual repeated activity that induces long-term metabolic and cardiovascular adaptations. The benefits of chronic exercise training include decreased ROS and pro-inflammatory cytokines with a concomitant increase in anti-oxidants and anti-inflammatory cytokines. It is still whether. The decrease in oxidative stress is a result of both reduced free radical production and from an increase in the antioxidant defense system. However, there seems to be an active stress-strain relationship between the formation and scavenging of oxidants during exercise.

### ***Oxidative Stress:***

A study by Bloomer et al (80), found that healthy, trained subjects had lower levels of oxidative stress at rest. Most studies demonstrate the reduction in post-exercise oxidative stress in chronically trained subjects (52, 81, 82). Exercise training is also able to reduce disease-induced increases in lipid hydroperoxides (83, 84), protein carbonylation (83), and vascular function (84). In a rat model, exercise training was able to reduce oxidative stress in various tissues, such as brain, liver, and muscle; indicating that the effects of exercise involves a system-wide response (85). A possible mechanism for the reduction in oxidative stress is the upregulation of nitric oxide and antioxidants, as explained below.

### ***Antioxidants.***

Over an extended period of exercise training, cells may activate *de novo* synthesis of antioxidant enzymes to manage oxidative stress. SOD, as one of the initial antioxidants to respond to oxidative stress, has often been reported to increase in response to exercise training (86–88). Catalase activity in skeletal muscle in response to exercise training has inconsistent results. Some authors show an increase (87, 88), a decrease (86, 89), or no change (90). The

activity of GPX demonstrates a muscle fiber-specific pattern with type 2a being the most responsive to training (86, 91).

In animal models, Vitamin E was shown to decrease in skeletal muscle, specifically fast twitch muscle (85), liver, and heart (92, 93). Vitamin E supplements were shown to increase resistance to exercise-induced lipid peroxidation (94, 95). Non-enzymatic enzymes are also altered in other tissues. In rats, ascorbic acid was shown to increase in the brain, but decreased in fast and slow twitch muscle. A-tocopherol was shown to decrease in fast muscle, while ubiquinone decreased in slow muscle (85).

### ***Inflammation.***

Leisure-time physical activity, as assessed by questionnaires, was found to be inversely related to levels of inflammation (96–98), such as CRP, plasma fibrinogen concentration, white blood cell count, and adhesion molecules. In addition, physical activity was also inversely associated with cytokines such as TNF and IL-6 (99). Maintenance of physical activity over a 10-year follow-up was associated with lower CRP and IL-6 levels, independent of adiposity (100). However, questionnaires can be misleading, as subjects can easily over- or underestimate their true physical activity intensity. One study, which did not find a relationship between fitness questionnaires and CRP, did find that peak oxygen uptake was inversely related to CRP (101). This coincided with other studies of physical fitness, as assessed by fitness tests that observed an inverse relationship was found with markers of CRP (102–104), fibrinogen and white blood counts (103).

## II.5 Conclusion.

Oxidative stress and inflammation are generated naturally in the animal and human body, and remain in balance due to a compensatory response of anti-oxidants and anti-inflammatory cytokines, respectively. However, under certain conditions such as disease, aging, and even acute exercise, this balance can be disturbed. This section demonstrated that exercise training can reverse an oxidative or inflammatory environment to a healthy, homeostatic level.

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# **Chapter III:**

**Sickle Cell Disease**

**&**

**Sickle Cell Trait**

## Chapter III: Sickle Cell Disease and Sickle Cell Trait

### III.1 Sickle Cell Disease

#### III.1a Pathogenesis

Sickle cell disease (SCD) is a cyclic disease which results from a single mutation in the  $\beta$ -globin chain inducing the substitution of valine for glutamic acid at the sixth amino acid position (1). Those present with both



Figure 7: Sickled cell vs normal HbA hemoglobin.

hemoglobin A (HbA) and HbS carry sickle cell trait, while the homozygous carriers

(HbSS) have sickle cell disease. Sickle cell disease is most often found in those of African descent (1, 2) although individuals of Mediterranean, Caribbean, South and Central America, and Arab descent are also affected due to migration (2). The average life span for men and women with sickle cell disease in the United States is 42 and 48 years, respectively. There is limited data on the mortality rate of those on the African continent, although it is estimated that half of those with sickle cell disease die before the age of five (2).

The pathogenesis of sickle cell disease occurs due to the polymerization of deoxygenated hemoglobin S. A typical red blood cell is continuously oxygenated and deoxygenated as it passes, about four times per minute, through the lung (3). During the oxygenated period, there is relatively little difference between HbA and HbS when oxygen is attached to the hemoglobin. It is during the period of deoxygenation that reversible polymerization could occur (1, 3). This polymer formation alters the normal biconcave disc shape into a rigid,

irregular shaped, unstable cell (1). When the HbS returns to the lungs, the picked up oxygen covers the reactive sites and the blood cell resumes acting like HbA. See Figure 8.

Due to the repeated polymerization and depolymerization of HbS, erythrocytes become rigid and destabilize, causing intravenous hemolysis to release cell free hemoglobin into the plasma (3). This incident causes a cyclic cascade which can lead to blood cell adhesion, vaso-occlusion, and ischemia-reperfusion injury. It is in this context that the levels of reactive oxygen species and anti-oxidants are altered, sometimes causing the events, other time produced because of the events. The manifestation of these actions produces secondary disease states such as acute chest syndrome, pulmonary hypertension, and stroke.

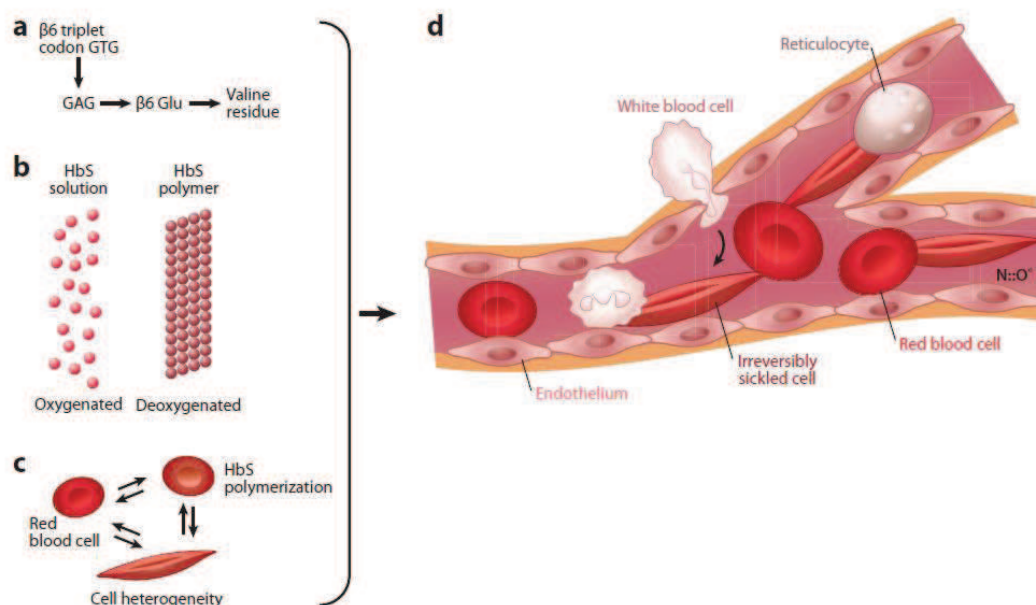


Figure 8: Pathophysiology of vaso-occlusion. (a) gene mutation, (b) polymerization of HbS during deoxygenation, (c) change in RBC shape due to polymerization, (d) cells in circulation (4)

### III.2 Role of Oxidative Stress (Published Review: See Annex IX.1a)

Sickle cell disease causes harmful pathophysiological effects, including sickling, vaso-occlusion, and ischemia-reperfusion injury. These complications have a cyclic nature which involves the generation and impairment of oxidative stress. Studies have shown relationships between markers of oxidative stress and common secondary diseases in SCD, such as acute chest syndrome and pulmonary hypertension (4, 5). Although there is elevated oxidative stress in SCD, these markers can also be mediated by other outside factors such as diet, physical activity, environment, and other co-morbid diseases associated with SCD. Therefore, the use of oxidative stress as a potential marker of disease severity has yet to be fully studied.

#### III.2a. Blood Cell Auto-oxidation

The intracellular polymerization of HbS during deoxygenation is the primary pathogenetic event in SCD. Polymerization can transform a normal red blood cell into a dense, inflexible blood cell. The rate of polymerization has been shown *in vitro* to be correlated with the concentration of HbS and with the cell-free heme released after autoxidation (6, 7).

The RBC reoxygenation phase is a major source of free radical production in sickle cell disease. During this period normal red blood cells can generate a significant amount of superoxide due to an electron transfer between the heme iron and oxygen. In the presence of oxygen, heme auto-oxidizes inducing methemoglobin and superoxide formation. Although both HbA and HbS blood have a tendency to autoxidize into methemoglobin and superoxide (8, 9), some studies show that HbS can auto-oxidize 1.7 times faster than HbA (10, 11), while others show a comparable rate (12). Unlike HbA which can counter this reaction to form

harmless byproducts, HbS can become overwhelmed by the continual source of superoxide and, via its dismutation, hydrogen peroxide (8). The formation of hydrogen peroxide, when exposed to methemoglobin, decomposes hemoglobin and releases iron. This iron can then react with remaining hydrogen peroxide to further produce hydroxyl radical, the most reactive and harmful of the reactive species (8). Sickled cells ultimately generate about 2-fold greater quantities of superoxide, hydrogen peroxide, and hydroxyl radical than HbA (13).

### III.2b. Sickling & Hemolysis

The sickled RBC often ruptures during its transport through the blood vessels. Hemolysis, along with the consequence of repeated sickling and unsickling, causes the premature destruction of erythrocytes (9). Studies have shown that sickled erythrocytes have a decreased half-life (14); only surviving for 10 days (14) compared to 50 days in normal red blood cells (10). Because of their short half-life, there is a rapid turnover of red blood cells, resulting in an increased proportion of reticulocytes. Present in these young cells is a high concentration of arginase, which can also be released into the plasma during hemolysis (15). About 1/3 of the destruction occurs within the vessels, releasing several grams of hemoglobin and iron (10) into the plasma.

Sickle cell disease patients have higher levels of hemolysis than healthy subjects, although the only true marker of hemolysis is actual measurement of the blood cell lifespan. Uncorrected reticulocyte count, as a measure of RBC turnover, is a secondary option (16). In a study looking at SCD patients in comparison to healthy volunteers, it was found that plasma arginase concentration, a biomarker for hemolysis, was significantly higher in SCD patients and correlated strongly with the concentration of plasma-free hemoglobin (5). Other clinically-used markers such as bilirubin and lactate dehydrogenase (LDH) are a less accurate



measure (16). This increase in the rate of hemolysis is a starting point for many of the subsequent complications of SCD, especially considering its involvement with nitric oxide.

### III.2c. Fate of Nitric Oxide

Nitric oxide is an important regulator of vascular tone, blood flow, and adhesion. In sickle cell disease patients, not only is the generation nitric oxide dramatically decreased, but the amount available for use is limited as well. Its bioavailability can be reduced in several ways: 1) via increases in the free radical superoxide; 2) through the products of hemolysis (free heme and arginase); and 3) through the “uncoupling” of eNOS (See Figure 9).

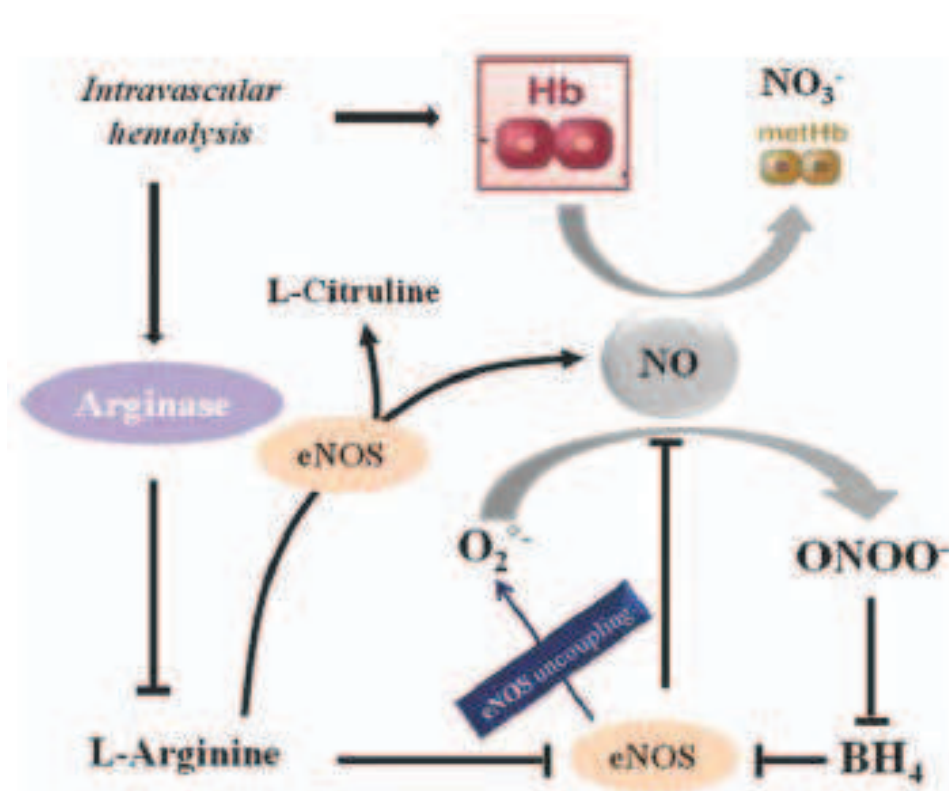


Figure 9: Degradation of NO. NO is decreased in three ways: through the reaction with O<sub>2</sub><sup>-</sup> forming ONOO<sup>-</sup> and through its inhibition via the byproducts of hemolysis

The interaction between superoxide and nitric oxide is more detrimental than their individual actions. While in normal systems superoxide can easily be dismutated by SOD, the overproduction of  $O_2^{\cdot -}$  seen in SCD overwhelms the body's defenses and reacts at diffusion-limited rates with nitric oxide. The reaction of NO and superoxide results in the generation of peroxynitrite ( $ONOO^-$ ): a powerful and highly reactant oxidant. The generation of  $ONOO^-$  is favored over spontaneous superoxide dismutation and NO autoxidation; the interaction is faster than both NO's reaction with hemoglobin and superoxide's reaction with SOD (8).  $ONOO^-$  has the opportunity to form hydroxyl radical and nitrogen dioxide ( $NO_2$ ); two other potent oxidants (8). The reaction between NO and  $O_2^{\cdot -}$  is two-fold in that it not only further decreases the concentration of NO, but also produces more reactive free radicals.

Cell-free hemoglobin has a large impact on the bioavailability of nitric oxide, resulting in endothelial dysfunction and vasoconstriction. Hemolysis increases the concentration of plasma hemoglobin, which allows for the reaction of both deoxy- and oxy-HbS with NO. The increased propensity for this reaction leads to the decrease in NO bioavailability. The binding of NO to deoxygenated hemoglobin results in the formation of a stable  $Fe^{2+}Hb-NO$  complex, which can readily react in Fenton reactions (17). The reaction between NO and oxygenated hemoglobin can form methemoglobin and nitrate. This becomes problematic in SCD because it has been reported that these patients have a much higher level of cell-free hemoglobin and methemoglobin leading to the consumption and decreased bioavailability of NO (15, 18–21). It has been shown that SCD patients have up to 20  $\mu M$  of heme in blood compared to the normal level of 0.2  $\mu M$ . During episodes of crisis, this number can increase several fold (19). A study by Reiter and Gladwin found that in sickle cell disease patients, those with plasma heme concentrations of 6  $\mu M$  or greater had a decreased NO response by 80% (18). Even with

levels as low as 1  $\mu$ M, the amount of NO able to reach smooth muscle cells was significantly reduced, concluding that during crisis plasma-free hemoglobin levels were high enough to sufficiently deplete NO bioavailability (19). The effect of NO scavenging via cell-free hemoglobin has a role in increasing vasoconstriction and decreased oxygen delivery through the abatement of NO-dependent vasodilation (22).

Nitric oxide is further reduced through the consequence of endothelial nitric oxide synthase (eNOS) “uncoupling”. eNOS is an enzyme made up of a reductase domain and an oxygenase domain which produces NO. Normally through the catalytic action of eNOS, tetrahydrobiopterin (BH<sub>4</sub>) transfers an electron in the oxygenase domain, converting L-arginine into nitric oxide and L-citruline. However, under certain conditions, eNOS can produce superoxide rather than NO. The two main co-factors of this mechanism, L-arginine and BH<sub>4</sub>, are reduced due to previous repercussions of SCD, therefore generating superoxide (20, 23).

L-arginine concentrations are decreased in SCD, and because it is the rate-limiting substance in NO synthase, can uncouple eNOS and generate additional superoxide (24). Arginine is considered a major target in hemolysis because its concentration can be diminished by arginase, one of the products of hemolysis. Arginase can out-compete the actions of eNOS by converting L-arginine into ornithine and urea. A deficiency of L-arginine interrupts the electron flow through the eNOS domains and favors the generation of superoxide over nitric oxide (23). In some SCD patients, L-arginine levels are significantly decreased (20), coinciding with an almost 2-fold increase in arginase levels (15, 20, 25); resulting in a significant decrease in NO bioavailability. The important role of L-arginine in NO production has been demonstrated with the supplementation of L-arginine in the diet of both mice and humans. This treatment was shown to reverse NO resistance (26), increase NO

bioavailability, and increase antioxidant activity (27). These studies demonstrate a tight association between the decrease in arginine due to hemolysis and nitric oxide levels.

Another important co-factor of eNOS, tetrahydrobiopterin ( $\text{BH}_4$ ), is also affected by the repercussions of SCD, leading to superoxide generation. A deficiency of  $\text{BH}_4$  can lead to the production of superoxide via the “uncoupling” of eNOS (23).  $\text{BH}_4$  can be inactivated by  $\text{ONOO}^-$  (23, 28), which is over abundantly produced in SCD, via the reaction between NO and  $\text{O}_2^-$ . In this situation, similar to L-arginine, eNOS functions as a producer of superoxide rather than nitric oxide (23). *In vitro*, human, and animal studies have demonstrated the link between  $\text{BH}_4$ ,  $\text{ONOO}^-$ , and superoxide in eNOS-producing NO. In cultured endothelial cells, a deficiency of  $\text{BH}_4$  was shown to uncouple eNOS, while its supplementation completely restored eNOS activity (29) and increased NO production (29). The  $\text{BH}_4$ -reducing capabilities of  $\text{ONOO}^-$  was further demonstrated in human where  $\text{ONOO}^-$  increased 8-F2-isoprostanes (30) and supplementation of  $\text{BH}_4$  recoupled eNOS, increased NO production, and inhibited superoxide (29). Sickle transgenic mice demonstrated a decrease in  $\text{BH}_4$  levels responsible for eNOS uncoupling (23).  $\text{ONOO}^-$  increased the production of  $\text{O}_2^-$  in the vessels of control mice in contrast to eNOS deficient mice, while increasing aortic lipid peroxides in rats (30). The combined effects of these studies demonstrate the vital role of  $\text{BH}_4$  in assisting eNOS function.

### **III.2d RBC Adhesion & Vaso-occlusion**

In addition to the decreased bioavailability of NO which can reduce vasodilation, a variety of adhesion molecules expressed on sickled erythrocytes can also impair blood flow. Under normal circumstances, circulating blood cells loosely come into continuous contact with the endothelial cells of blood vessels. In conditions such as SCD, there is an increase in

adherence to the vessel walls. Activation of vascular endothelial cells and circulating blood cells represent the continual inflammation seen in SCD. Upon activation, circulating white blood cells and platelets express adhesion glycoproteins. Consequently, endothelial dysfunction is modulated by the interaction between blood cells and platelets and the cellular and molecular components in the endothelium. In this context, blood cell adherence to the endothelium can be modulated by factors such as decreased NO bioavailability, hemolysis, ROS, and inflammation. This abnormal interaction involves adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), E-selectin, and P-selectin, which are overexpressed in sickle cell disease (23, 31–34). Because of this over expression, sickled red blood cells are at least 2.5 times more likely to adhere to endothelial cells than normal red blood cells (32, 35). VCAM-1 and ICAM-1 are associated with decreased NO bioavailability (31–33) and increased hemolysis (25). Levels of adhesion correlate positively with LDH (31) and arginase (20), often used markers of hemolysis (25); In both human and cross-species studies, sickled red blood cells incubated with a NO donor decreased adhesion (32). These results suggest that a decrease in NO bioavailability and an increase in hemolysis can assist in furthering blood cell adherence.

Oxidative stress can promote sickled blood cell adherence to the endothelium, while the supplementation of antioxidants can reduce the expression of adhesion molecules. The interaction between sickled red blood cells and endothelial cells is associated with a 3-fold increase in oxidative stress, as measured by TBARS (35). The increase in TBARS was inhibited by the addition of SOD and catalase, which lead to a 50% decrease in lipid formation (35). Additions of the anti-oxidant N-acetylcysteine (NAC) inhibited the expression of ICAM-1 induced by  $H_2O_2$  (36), while the addition of a different anti-oxidant pyrrolidine dithiocarbamate inhibited the expression of VCAM-1 (37). The increase in oxidative stress is

linked to the activation of Nuclear Factor- $\kappa$ B (NF- $\kappa$ B) (35) as its expression is increased during periods of adhesion (35). NF- $\kappa$ B can alter the gene expression of VCAM-1, ICAM-1, and E-selectin, leading to their increase (38). An inhibitor of NF- $\kappa$ B, sulfasalazine, has been observed to decrease endothelial cell expression of VCAM-1, ICAM-1, and E-selectin in both humans and mice (34). NF- $\kappa$ B can also be regulated by tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ). These inflammatory cytokines are raised in SCD patients and can influence the expression of adhesion molecules as well in SCD patients (31). In both human and cross-species studies, normal cells enhanced with TNF- $\alpha$  increased adhesion, while the use of a NO donor abrogated this increase (32). Lipolate downregulated the TNF- $\alpha$  -induced expression of ICAM-1 and VCAM-1 (39). The link between the expression of cytokines and oxidative stress is further verified through the addition of antioxidants. The inhibition of NF- $\kappa$ B induced adhesion via antioxidants demonstrates the interaction of oxidative stress on adhesion.

Adhesion of sickled RBC to the endothelium and other blood cells, as well as pre-capillary obstruction by rigid, sickled blood cells can initiate vaso-occlusion in the microvasculature (40). This results in vaso-occlusion crises, which include recurrent episodes of severe pain, in patients with SCD. The frequency of vaso-occlusive episodes was reduced 5-fold due to the administration of NAC (41), which was additionally shown to inhibit ICAM-1 expression (36). The vessel occlusion causes tissue ischemia in downstream areas deprived of oxygen. Eventually these venules reestablish blood flow, leading to reperfusion injury (42).

### III.2e. Hypoxia/Reperfusion Injury

A common repercussion of sickle cell disease due to adhesion and vaso-occlusion is the occurrence of hypoxia. The cessation of blood flow to tissues causes an ischemic or hypoxic environment. During this state, the limited concentration of oxygen available to tissues results in an inadequate amount of nutrients delivered to support the metabolic needs. Reactive oxygen species can be generated at various points during this undesirable environment: during hypoxia in both the mitochondria and in tissues, as well as during the reperfusion phase that follows. This phase can cause reperfusion injury, referring to the damage caused to the vessel and tissue when oxygen is reintroduced to the tissues, leading to an increase in the concentration of radical species (43).

Under hypoxic conditions, as seen in SCD, adenosine triphosphate (ATP) is consumed to adenosine diphosphate and adenosine monophosphate. If oxygen supply continues to decrease to certain levels, adenosine monophosphate is catabolized, leading to the accumulation of hypoxanthine and xanthine in the tissue (42, 43). The produced xanthine oxidase (XO) can then lead to deleterious effects due to the restitution of blood flow and therefore oxygen to the cells. It is when oxygen is reintroduced to these tissues (“reoxygenation”) and reacts with the hypoxia-formed XO, that the damaging effects of oxidative stress are seen. This reaction results in the conversion of hypoxanthine and xanthine by XO into superoxide (38, 42, 43) (See Figure 10).



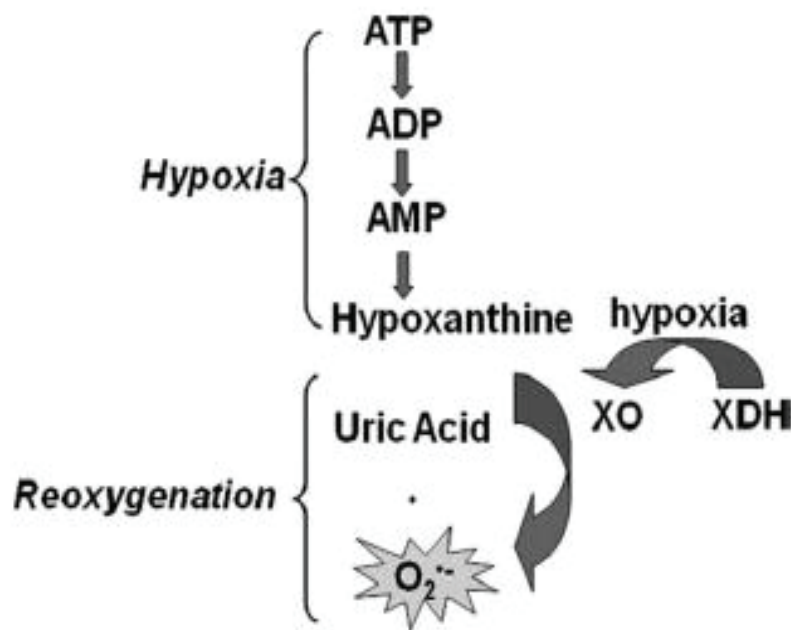


Figure 10: Hypoxia/reoxygenation phenomenon. Under conditions of hypoxia, hypoxanthine and XO are generated. During reoxygenation, these two products can be converted into superoxide.

A study by Osarogiagbon et al on reperfusion injury in sickle mice found that the mice who were subjected to hypoxic conditions showed a significantly higher proportion of XO than both control mice and sickle mice in ambient conditions (42). As supported by the equation above, this increase in hypoxia-generated XO can lead to the production of  $O_2^{\bullet-}$ . The increase in  $O_2^{\bullet-}$  can lead to a chain reaction of free radicals as observed by the increase in ethane excretion and hydroxyl radicals, two markers of oxidative stress. To further verify that the increase in oxidative stress was indeed a result of XO, administration of allopurinol, an XO inhibitor, was shown to diminish ethane excretion levels (42). An increase in XO has been also associated with inflammatory cytokines such as  $TNF\alpha$ ,  $IL-1\beta$ , and  $IFN\gamma$  in many different tissues and diseases (44–46). As discussed in the previous section, there is a strong



correlation between oxidative stress and inflammatory cytokines. The cyclic nature of this disease is evident as the ROS-induced cytokines which can cause adhesion and hypoxia are also produced as a result of hypoxia-induced O<sub>2</sub>- production.

Hypoxia causes an increased proportion in the amount of sickled cells (42), possibly due to the sickle-inducing extended deoxygenation period brought about by the decreased oxygen (3). Conditions of hypoxia can also further increase the adhesive interactions within the vessel walls, exacerbating vaso-occlusion. Setty and Stewart demonstrated that sickled RBC, contrary to normal cells, exposed to hypoxia have a 66% greater risk of adhering to both the macrovasculature (as seen in the aorta) and the microvasculature (as seen in the retina) (33). It was shown that hypoxia regulates the production of VCAM-1 and ICAM-1, although VCAM-1 is solely responsible for the adherence of sickled cells to the endothelium (34). This regulation could be due to NF- $\kappa$ B, which is abundantly observed after hypoxia (42, 47), and can alter the expression of these adhesion molecules. See Figures 10 and 11.

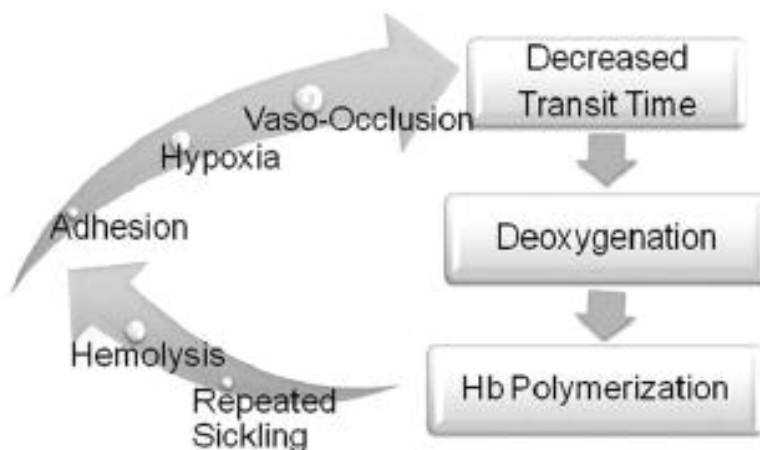


Figure 11: Vicious circle of SCD. The cyclic reaction beginning with SCD leading to the downstream effects which cycle back to restart the complications of the disease.

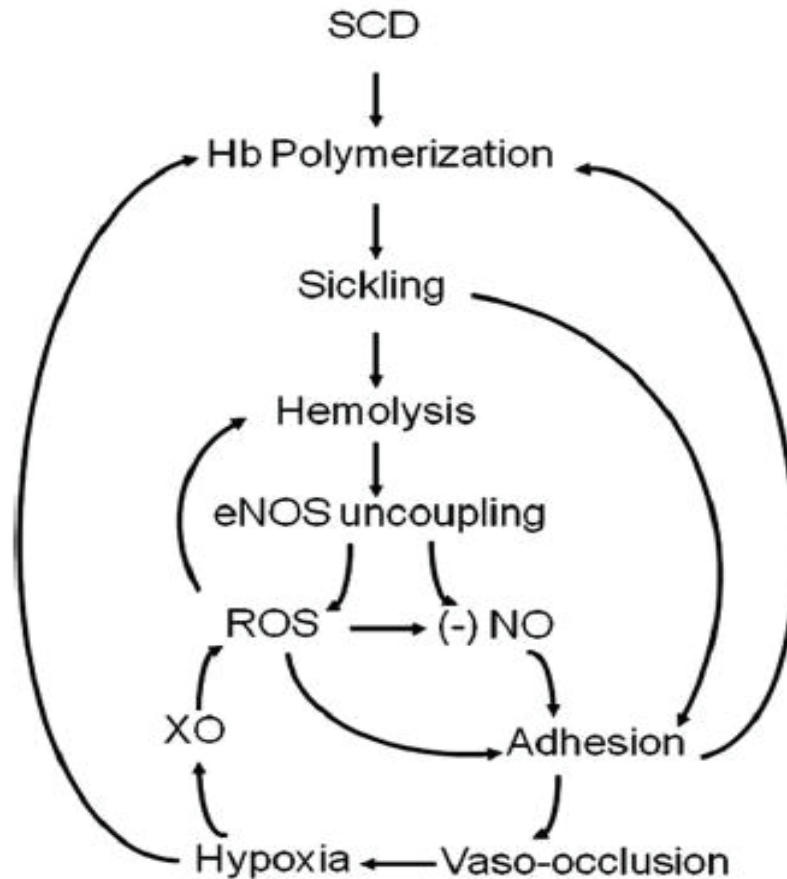


Figure 12: Cyclic reaction of RBC sickling. The normal oxygenation/deoxygenation process occurring in the lungs can be disturbed by an increase in adhesion, hypoxia, or vaso-occlusion. This can lead to increased RBC sickling and hemolysis, continuing the cycle.

### III.2f Anti-oxidants

In addition to the oxygen species that are formed in sickle cell disease, the protective mechanisms such as antioxidants are decreased. Those that provide enzymatic defense, including superoxide dismutase, glutathione peroxidase, catalase, and heme oxygenase-1, and those that scavenge free radicals, such as glutathione, vitamin C, and vitamin E, are most affected (48).

Superoxide dismutase has been shown to increase in some studies of sickle cell disease (49, 50), while others show decreased levels (27, 51). A study by Das and Nair (51) demonstrated an increase in SOD in RBCs of SCD patients concluding that this may be a defense mechanism due to the increase in oxidative stress, and could possibly cause a buildup of  $H_2O_2$ . A contrasting study by Schacter et al (51) showed that SOD levels decreased in proportion to disease severity in HbS patients compared to healthy black volunteers. This difference was suggested to be due to a more rapid degradation of SOD, possibly from oxygen radicals. They also showed that black controls have a naturally higher level of SOD than white controls; meaning that the main discrepancy between the studies could lie in the control subjects used (51).

Under normal circumstances,  $H_2O_2$ , which is either produced via a 2 electron transfer or due to sickling, is removed by 2 methods: glutathione peroxidase (GPX) or catalase. Due to the decreased levels of these enzymes in sickle cell disease patients (5, 8, 50), the endogenously produced  $H_2O_2$  cannot be sufficiently cleared (8).

GSH, a cofactor for GPX, is easily oxidized to glutathione disulfide (GSSG) through its reduction of free radicals and ROS, and is an essential element in order for GPX to reduce  $H_2O_2$ . GSH concentration is significantly reduced in sickle cell patients (13, 27), with some studies noting a 50% decrease in HbS compared to HbA (52). GPX concentrations are also reduced (48), with a direct relationship to the severity of the disease (27). A study by Morris et al. found that sickle cell disease patients had significantly lower concentration of total glutathione (GSH + GSSG) in the plasma and within the red blood cell compared to healthy patients (5). These results suggest that the overabundance of oxidative stress leads to consumption or inactivation of these protective elements.

There is a discrepancy between studies on the levels of catalase in sickle cell disease; a study from Dasgupta et al has shown decreased levels (27), whereas another from Das and Nair has observed increased levels in patients (50). The increase in catalase could be a protective effect in order to scavenge  $H_2O_2$ , while the decreased levels could be due to the overwhelming concentration of oxidative stress.

Heme oxygenase-1 (HO-1) is a cytoprotective enzyme that is activated in response to heme-induced oxidative stress. HO-1 can inhibit NF- $\kappa$ B, VCAM-1, ICAM-1, leukocyte-endothelial interactions, and hypoxia-reperfusion induced stasis. In addition, the byproducts of HO-1, including carbon monoxide, biliverdin and iron, have antioxidant and anti-inflammatory properties. Although humans and transgenic mice have been shown to upregulate HO-1 in response to heme-induced oxidative stress, SCD patients have insufficient activity to handle the burden of heme (17). SCD patients are unable to activate HO-1 in response to prolonged hemolysis, preventing the inhibition of oxidative stress, vaso-occlusion, and hypoxia/reperfusion injury (17).

### III.2g Anti-oxidant therapeutic strategies

Several treatment studies have been shown to be effective to reduce pathological consequences of the disease (See Wood et al (10); Nur et al (53) for review). Susceptibility to peroxidation, due to an increase in ROS or during conditions of reduced oxygen, can increase 3-fold in sickle cell patients. *In vitro*, pre-incubation of sickle erythrocytes with vitamin E decreased the susceptibility to peroxidation. This finding is maintained *in vivo*, where those with a vitamin E deficiency increased levels of peroxidation compared to control subjects (54). A study evaluating the effects of SCD by Natta et al, found that after only 10 weeks of vitamin E supplementation, the percentage of irreversibly sickled red cells decreased from

25% to 11% (55). A decrease in sickled RBC was also observed during a supplementation period with vitamin E possibly due to the almost 2-fold decrease of ROS and the 1.2-fold increase in the concentration of GSH (13).

The additions of ascorbic acid and dehydroascorbic acid supplements were able to inhibit dense RBC formation and lipid peroxidation levels in sickle cell patients (56). A another study by Amer et al found that supplementation of vitamin C helped to decrease ROS production almost 4-fold while increasing the concentration of GSH almost 2-fold (13). It may be through this mechanism that ascorbic acid supplements were shows to prevent H<sub>2</sub>O<sub>2</sub>-induced hemolysis (13). Finally, *in vitro* supplementation of alpha-lipoic acid, known to have potent antioxidant properties, can inhibit RBC sickling by 50% (56), decrease oxidation (57), protect peroxy radical-induced hemolysis, and increase GSH synthesis (58).

Taken together these promising results should encourage the development of such antioxidant therapy. However the conclusions of clinical trials using antioxidant therapeutic treatment in SCD are not so enthusiastic (23). Wood et al (23) suggest that the SCD-induced ROS generation may greatly overwhelm the capacity of the exogenous antioxidants, leading to the conclusion that a cocktail of multiple antioxidants may be more efficient (56).

Therapeutic strategies that focus on decreasing ROS production, instead of increasing their neutralization, were also recently studied. Hydroxyurea (HU) can reduce the occurrence of vaso-occlusive crises and pulmonary events by inducing fetal hemoglobin. However, in addition to fetal hemoglobin, HU can limit ROS production and NO scavenging via a decrease in hemolysis. To determine mechanisms, *in vitro* studies have found a decrease in adhesion between HbS RBC and the endothelium, as well as a decrease in adhesion molecules on sickle cell reticulocytes (59). Some studies have suggested that HU induces a NO response

(60), as NO is well documented in the regulation of adhesion. Because of the importance in maintaining proper NO levels, exogenous NO treatment is often beneficial. Inhaled NO was shown to increase plasma NO<sub>x</sub> in some case studies, and decrease adhesion and ischemia/reperfusion injury in animal studies (10). Arginine therapy is shown to have a positive effect on vasodilation, possibly through the reduction of oxidative stress and hemolysis. Kaul et al (26) suggested that arginine therapy in SCD mice prevented the oxidative stress-induced hemolysis of RBC. An increase in the antioxidant GSH and enhanced NO production was also observed in these mice, possibly due to a reversal of eNOS uncoupling (26).

Iron chelator desferoxamine (61), a catalase mimetic (38) and an NF- $\kappa$ B inhibitor (62) were shown to attenuate oxidative stress, adhesion and inflammation in murine models of SCD. Although not tested yet in SCD, the use of SOD mimetic (such as tempol) may potentially be beneficial to target the anion superoxide clearly identified in the pathogenesis of SCD (23).

### III.3 Role of Exercise

#### III.3a Exercise in SCT

Sickle cell trait (SCT) is the heterozygous form of SCD and is characterized by the presence of both normal hemoglobin (HbA) and sickled hemoglobin. SCT is usually considered to be benign and asymptomatic as numerous studies have reported normal growth and development, morbidity, and mortality (63). However several authors have suggested that it should be reclassified as a disease state (64, 65). In a rebuttal position statement, Connes (64) stated that metabolic or environmental changes such as hypoxia, acidosis, or dehydration could be responsible for the change from a silent condition into one resembling sickle cell disease with vaso-occlusive crisis resulting from an increase in low deformable RBCs in the microcirculation. Furthermore, numerous studies have reported exercise-related sudden deaths of SCT carriers (63, 66–68); possibly due to RBC abnormalities associated with an increase in oxidative stress (50, 69).

Most carriers of sickle cell trait are able to perform exercise and sport as normal athletes would (70). However, because of the high incidence of sudden death in army recruits and athletes, it is important to understand how an acute bout of exercise may complicate the disease (See Editorial, Annex IV.1c)

#### *Acute Exercise in SCT*

Cardiopulmonary changes. SCT has little effect on aerobic metabolism or function. Most studies have found few differences between SCT carriers and control subjects in terms of cardiac function, such as cardiopulmonary exchange, oxygen deficit, oxygen consumption

(VO<sub>2</sub>), or mean aerobic power (MAP), at maximal aerobic levels (71–75). However, in the context of a constant heavy exercise period, it was shown that SCT carriers had a higher VO<sub>2</sub> slow component and a higher heart rate throughout the exercise protocol and recovery. This study indicates that SCT carriers may not be affected during the beginning stages of exercise, but are prone to exercise intolerance and a lower aerobic capacity as the exercise duration continues (76). Several other studies support this hypothesis postponed exercise intolerance (77, 78). A study looking at repeat maximal cycling sprints found that although peak power output and total work did not differ between SCT carriers and controls, SCT carriers experienced a decrease in sprint work, total work, and peak power output sooner than control groups. Although maximal anaerobic performance during a single sprint bout was not affected, the ability to repeat sprint bouts was decreased in SCT (79).

Hemorheology. Noticeable differences are evident in whole-blood rheology between SCT carriers and control subjects at rest and during exercise. In resting conditions, SCT carriers experience lower RBC deformability (71, 80), increased RBC aggregation, and high blood viscosity (80) compared to control subjects. However, no change is observed in hematocrit levels (80, 81). These results are in contrast with Monchanin et al<sup>12</sup> that found no change in blood or plasma viscosity during baseline. However, when corrected for hematocrit, blood viscosity was significantly higher in SCT carriers than control subjects at rest. Concerning the hemorheological responses of SCT carriers during exercise, it was found that SCT carriers have higher levels of RBC rigidity during an acute bout of exercise and remain high throughout recovery (71, 80, 82, 83). Blood and plasma viscosity and RBC disaggregation increase, while hematocrit and RBC deformation decrease in response to exercise and during recovery (71, 84). These combined results could indicate that SCT carriers experience disturbed hemorheological responses and could be at risk for microcirculatory disorders.



Alterations in hemorheology could be related to hydration status, potentially playing a role in microcirculatory and vascular alterations (70). In two studies by Diaw et al (85) and Tripette et al (80) comparing exercise in a hydration or dehydration condition, it was found that exercise in a dehydrated state elevated blood viscosity immediately after exercise (85) and throughout recovery (80), probably due to a decrease in RBC deformation (80). In addition, a marker for the efficacy of RBC oxygen transportation was lower at rest in both hydrated and dehydrated SCT groups, yet remained lower with exercise in the dehydrated group (85). Both studies found that the addition of water ad libitum standardized the hemorheology levels to those similar to control subjects (80, 85).

Adhesion. Few studies have studied the role of adhesion on SCT; however it appears that SCT carriers have an impaired adhesive response. In terms of selectin levels, SCT carriers were reported to have higher levels of p-selectin at baseline, in response to exercise, and throughout recovery (84), while a similar increase in l-selectin was seen during recovery (84, 86). A comparable pattern was seen with VCAM-1, as SCT carriers had higher levels at baseline (87, 88). Although both SCT carriers and control subjects experienced an exercise-induced increase in VCAM-1, only SCT carriers remained high throughout recovery (87, 88). This is in contrast with another study by Tripette et al (86) that found no change in either VCAM-1 or ICAM-1 at rest or in response to exercise. It is unclear as to the reasons behind this contradiction as it was probably not due to the exercise protocol. In the two studies by Monchanin et al where SCT carriers experienced higher VAM-1 levels, participants performed a maximal exercise test with (88) or without (87) an additional submaximal endurance test, while in the two studies by Tripette et al where SCT carriers experienced no change in VCAM-1 levels, participants performed a submaximal endurance test (84) or three

repeated maximal exercise tests (86). Therefore, it seems that this response is unrelated to the intensity or duration of the exercise bout.

*Oxidative Stress and NO.* It is unclear whether oxidative stress and nitric oxide metabolism is impaired in SCT carriers. Although few studies have looked at changes in oxidative in this context, evidence suggests that there is little difference between SCT carriers and control subjects in response to exercise or during recovery (84, 89). Nonetheless, there was a slight trend towards increased susceptibility to oxidation in SCT erythrocytes during exercise (89). Contrary to this, Faës et al (90) found that SCT carriers had an increase in AOPP in response to exercise, while MDA remained higher during recovery than control subjects. In addition, no change in nitric oxide metabolism was observed between subject groups or in response to exercise (84, 90). In terms of antioxidant capacity, it was found that SOD and catalase was expressed similarly in both SCT carriers and control groups in responses to exercise and recovery (89, 90). However, glutathione peroxidase and two NADPH-generating enzymes, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, remained lower in SCT carriers and did not response to exercise as the control group did. This is in contrast with Faës et al (90) who found no difference between SCT and control subjects in terms of glutathione peroxidase. The lack of an induction of NADPH-generating enzymes due to exercise training in SCT carriers may explain the why SCT carriers have a slight susceptibility to oxidation. The difference in oxidative stress results could be due to the exercise intensities. In the studies by Das et al and Tripette et al, subjects performed a submaximal exercise test, while in the study by Faës et al (90), the subjects performed a maximal exercise test. As ROS production is known to be dependent on exercise intensity (91), maximal exercise may be required to observe oxidative stress and NO metabolism impairment as proposed for healthy (92, 93) and trained subjects (94). Therefore, it is

essential to test SCT carriers during high intensity exercise to determine the potential role of oxidative stress in sudden death in these subjects.

### *Chronic Exercise in SCT.*

There are only two transversal studies that looked at the effects of a training protocol on SCT carriers (95, 96). In these two studies, trained (at least 8 hours of sport training per week) and untrained SCT carriers were compared to control trained and untrained subjects. Various markers were evaluated in response to a maximal exercise test. In agreement with the acute exercise studies in SCT carriers, both studies found that  $HR_{max}$  was similar between all groups. As expected (97), MAP and relative MAP was higher in trained subjects compared to untrained subjects (95, 96).

No difference was observed in markers of inflammation such as Interleukins-4, 5, 8, and 10, and  $TNF\alpha$ . sICAM-1, sP-selectin, and sE-selectin were not difference between SCT carriers and control subjects; all groups experienced a similar pattern of increase in response to exercise. Levels of sVCAM-1 were elevated in untrained SCT but were not different within trained subjects. This is in agreement with the study by Tripette et al (84, 86) in which the trained subjects did not differ in terms of VCAM, but in contrast with the study by Monchanin et al (87, 88). As VCAM-1 is involved in the interaction between cells such as leukocytes and endothelial cells (98), an increase in VCAM-1 could result in an increased risk of vaso-occlusive events such as blood cell aggregation and thrombus formation (99). It can therefore be suggested that the lack of a difference between trained subjects and an increase in untrained SCT carriers indicates that regular exercise training may reduce vaso-occlusive events.

In a study by Vincent et al (96), it was shown that exercise trained SCT carriers have an altered oxidative potential compared to control subjects, as measured by the different mitochondrial respiratory chain complex content. Remodeling of the muscle microvascular network is proportionally maintained between trained and untrained SCT carrier and control subjects, as similar improvements are seen in capillary density and number of capillaries around a single fiber (96).

There are no studies that have looked at the relationship between exercise training in SCT and oxidative stress. A study involving pro-oxidant/anti-oxidant balance may help to understand the beneficial effects of exercise training in SCT subjects as i) many of the abnormalities of SCT (such as adhesion and inflammation) and the beneficial effects of exercise training are intimately linked with oxidative stress (see chapter II.4), and ii) exercise training is known to improve antioxidant enzymes (see chapter II.4).

### III.3b Exercise in SCD

It is uncertain as to whether physical activity is safe or effective in SCD patients (100). The presence of anemia may induce a faster transition from aerobic to anaerobic metabolism during exercise, leading to the polymerization and sickling of HbS (101). Dehydration and temperature change occurring during exercise is another concern which may contribute to RBC sickling (100).

#### *Acute Exercise*

Cardiopulmonary changes. Sickle cell disease patients exhibit altered cardiopulmonary reactions in response to exercise. In a study by Callahan et al (102), it was observed that although all SCD patients could perform a symptom-limited cardiopulmonary exercise test without complications, all expressed abnormal  $\text{VO}_2$  responses paired with at least a lower-

than-predicted peak  $\text{VO}_2$  or anaerobic threshold, lower  $\text{O}_2$  pulse, or lower  $\text{VO}_2$ -work rate ratio. This is in agreement with several other studies that observed that both children and adults experience reduced aerobic performance (103, 104), anaerobic power (101, 105), higher heart rate (101, 105), greater cardiorespiratory stress (105), and increased ventilation (105) in response to exercise.

Hemorheology. Studies examining a mild to moderate exercise test performed by steady state SCD patients found similarities between SCD patients and control subjects in terms of hemorheological and hematological changes. Several studies found that exercise did not change hematocrit, blood viscosity, or hemoglobin (104–106). However, one study found that the only change in response to exercise was that the proportion of intermediary dense cells and irreversibly sickle cells was higher at baseline and exercise increased the proportion of ISCs while reducing intermediary dense cells in SCD patients (105). Another found that exercise impaired RBC disaggregation during recovery, concurrent with platelet count diminution (106). Nonetheless, the overall results of these studies indicate that this intensity of exercise is not harmful for SCD patients and may benefit microcirculatory blood flow.

Adhesion. Adhesion has not been fully studied in SCD patients in response to exercise. In one study by Barbeau et al (104), SCD patients performed three repeated bouts of moderate-intensity exercise. It was found that SCD patients had higher levels of vasoactive mediators, endothelin-1 and nitric oxide, at baseline. Endothelin-1 was significantly higher than control subjects throughout the three days of exercise, but did not change significantly from pre- to post- exercise. At the end of day 3, nitric oxide metabolism was higher in SCD patients with no change throughout the protocol in control subjects. These results suggest that chronic exercise training may be necessary to induce a beneficial response to adhesion, as it was shown in SCT (95).

Inflammation. SCD patients exhibit increased inflammation which does not increase in response to exercise. Low-level chronic inflammation, as measured by TNF $\alpha$ , was observed at baseline in SCD patients (104). However, other studies found higher levels of inflammation, such as IL-6 and TNF $\alpha$ , in baseline conditions (47, 107, 108). In the only study measuring the response of inflammatory markers in response to exercise, it was found that throughout three repeated bouts of moderate-intensity exercise, TNF $\alpha$  remained higher in SCD patients than in control subjects. However, the pattern of change between exercise bouts and throughout recovery was similar between groups (104). This data suggests that even with an increased inflammatory response at baseline, exercise does not further modify this response.

### ***Chronic Exercise***

Little information is available on the role of chronic exercise in patients with SCD. One case study examined the effects of kinesiotherapy/aquatic rehabilitation over 5 weeks in a young adult with SCD. Using a series of questionnaires, the patient indicated a significant improvement in quality of life and pain management. In addition, the patient experienced improved respiratory pressures and reported experiencing less daily fatigue due to the exercise program (109). This study shows that exercise training is able to exert beneficial responses, both psychologically and physically, in a SCD patient. Nonetheless, this study only looked at one individual patient and can not be used to properly evaluate the effects of an exercise program in SCD.

Although there have been positive results in one case study, physicians are hesitant to recommend physical activity in SCD patients because of the increased risk of VOC (100). As a means to test the precise mechanisms within this disease without risking the health of patients, researchers are turning to transgenic SCD mice. A study by Aufradet et al observed

the effects of voluntary exercise training in SCD (SAD) mice on the response to an acute hypoxia/reoxygenation (H/R) stress. The study found that exercise training was able to reduce endothelial VCAM-1 lung expression and decrease oxidative stress in response to H/R stress in SAD mice. Exercise training alone was able to increase antioxidant activity in the heart and decreased LDH concentrations in SAD mice. These results demonstrate that an exercise-induced reduction in oxidative stress and adhesion may decrease the risk of vaso-occlusive crises.

**Summary.** In conclusion, SCT carriers experience impaired hemorheology and increased adhesion and oxidative stress in response to an acute exercise bout. However, SCT carriers that perform chronic exercise training respond similarly to control subjects, as little difference is noted in adhesion and muscle microvascular remodeling. Nonetheless, further studies will have to be performed in order to assess additional mechanisms that explain the beneficial effects of exercise training. Because of the role of oxidative stress and nitric oxide impairment in SCD, it could be valuable to examine the whether this mechanism is impaired in SCT carriers and whether exercise could reverse this susceptibility.

The complications associated with SCD are more severe than with SCT carriers. It is because of this that few studies have examined the role of exercise in these patients. Acute exercise testing has demonstrated an impaired cardiovascular and hemorheologic response paired with increased adhesion and inflammation. However low-intensity chronic exercise training could improve quality of life while decreasing the risk of vaso-occlusive crises. Because the beneficial effects seen in SCT carriers in response to exercise training could be well translated to SCD patients, it is important to carry out further studies examining other factors such as adhesion, inflammation, and oxidative stress in these subjects.

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## **Chapter IV**

### **Article #1:**

### **Exercise training blunts oxidative stress in sickle cell trait carriers**

## Chapter IV

### IV.1 Introduction to Article #1

As stated in the previous chapter, SCD causes cyclic pathophysiological events such as sickling, vaso-occlusion, and ischemia-reperfusion injury, which involves the generation of oxidative stress. As exercise training is known to reduce oxidative stress in many categories of disease, it seems that exercise training could be a viable method for controlling these symptoms leading to vaso-occlusive crisis. However as SCD is a highly complex disease with a high rate of mortality, many studies have focused on the less severe, heterozygous form of this condition: sickle cell trait (SCT). Although SCT is typically considered to be benign, sudden deaths have been reported to occur in response to heavy exertion and dehydration associated with exercise. It was shown that SCT carriers have a higher risk of exercise-related sudden death which is unexplained by any known preexisting condition (1). As suggested by Connes (2), abnormal blood rheology, hemostatic and vascular adhesion mechanisms paired with environmental factors may play a role in the incidence of these sudden deaths. It is also possible that oxidative stress may have a role in these fatal events (3). Because of this, the National Collegiate Athletic Association (NCAA) in 2010 began to require all incoming Division I athletes to be tested for SCT. This mandate is strongly controversial, and organizations such as the Sickle Cell Disease Association of America and the American Society of Hematology suggest further biomedical and epidemiological research on the pathophysiological implications of SCT.

The purpose of our first study was to determine how oxidative stress plays a role in SCT carriers: in response to exercise training as well as in response to an acute stress. Oxidative stress, which plays a large part in the pathogenesis of SCD, may also turn a “benign”

condition into a deadly one by increases the incidence of vaso-occlusive crises and deformed RBCs. The purpose and results of this study were not only deemed important by us, but also by Drs. Frans A. Kuypers and Anne M. Marsh who wrote an editorial highlighting the significance of this study (see Annex IX.1c).

In collaboration with University of Yaoundé, University of Saint-Etienne, and University of Grenoble, we set out to determine the effects of exercise training in SCT carriers. 40 male students from the University of Yaoundé with or without SCT participated in this group of studies. As it has been shown that metabolic changes such as dehydration, acidosis, or hypoxia could be responsible for the exercise-induced complications, an acute maximal exercise test was performed in order to elicit a stressful response. Blood that was collected immediately after the maximal exercise test and at various points throughout recovery was sent to our lab (CRIS) for analysis. The first study from our lab (Aufradet et al (4)), showed that the vascular markers of adhesion increased in SCT carriers and that exercise training is able to diminish this response. Because of the close alliance between markers of adhesion and oxidative stress, specifically nitric oxide, we shifted our attention toward the production of oxidative stress during chronic and acute exercise in SCT carriers. We looked at four markers of oxidative stress, three markers of anti-oxidants, and nitric oxide metabolism. As we expected to see the effects of chronic exercise training and the response to an acute maximal exercise test, we tested these markers immediately after a maximal exercise and during recovery. We found a beneficial response to exercise training in terms of adhesion and oxidative stress.

## IV.2 Exercise training blunts oxidative stress in sickle cell trait carriers

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**ABSTRACT**

The aim of this study was to analyze the effects of exercise training on oxidative stress in sickle cell trait carriers. Plasma levels of oxidative stress [AOPP (advanced oxidation protein products), protein carbonyl, MDA (malondialdehyde), and nitrotyrosine], antioxidant markers [catalase, GPX (glutathione peroxidase), and SOD (superoxide dismutase)], and NOx (nitrite and nitrate) were assessed at baseline, immediately following a maximal exercise test ( $T_{ex}$ ), and during recovery ( $T_{1h}$ ,  $T_{2h}$ ,  $T_{24h}$ ) in trained (T: 8 hours/week minimum) and untrained (U: no regular physical activity) sickle cell trait (SCT) carriers or control (CON) subjects (T-SCT,  $n=10$ ; U-SCT,  $n=8$ ; T-CON,  $n=11$ ; and U-CON,  $n=11$ ; age:  $23.5 \pm 2.2$  years). The trained subjects had higher SOD activities ( $7.6 \pm 5.4$  vs.  $5.2 \pm 2.1$  U/ml,  $p=0.016$ ) and lower levels of AOPP ( $142 \pm 102$  vs.  $177 \pm 102$   $\mu\text{mol/l}$ ,  $p=0.028$ ) and protein carbonyl ( $82.1 \pm 26.0$  vs.  $107.3 \pm 30.6$  nm/ml,  $p=0.010$ ) than the untrained subjects in response to exercise. In response to exercise, U-SCT had a higher level of AOPP ( $224 \pm 130$  vs.  $174 \pm 121$   $\mu\text{mol/l}$ ,  $p=.012$ ), nitrotyrosine ( $127 \pm 29.1$  vs.  $70.6 \pm 46.6$  nmol/l,  $p=0.003$ ), and protein carbonyl ( $114 \pm 34.0$  vs.  $86.9 \pm 26.8$  nm/ml,  $p=0.006$ ) compared to T-SCT. T-SCT had a higher SOD activity ( $8.50 \pm 7.2$  vs.  $4.30 \pm 2.5$  U/ml,  $p = 0.002$ ) and NOx ( $28.8 \pm 11.4$  vs.  $14.6 \pm 7.0$   $\mu\text{mol/l/min}$ ,  $p=0.003$ ) in response to exercise than U-SCT. Our data indicate that the overall oxidative stress and nitric oxide response is improved in exercise trained SCT carriers compared to their untrained counterparts. These results suggest that physical activity could be a viable method of controlling the oxidative stress. This could have a beneficial impact due to its involvement in endothelial dysfunction and subsequent vascular impairment in HbS carriers.

**Key Words:** Antioxidants, Exercise, Physical activity, Hemoglobin

## ABBREVIATIONS

AOPP: advanced oxidation protein products

CON: control

GPX: glutathione peroxidase

HbA: hemoglobin A

HbS: hemoglobin S

HbSS: hemoglobin SS

MAP: maximal aerobic power

MDA: malondialdehyde

NO: nitric oxide

NOx: nitric oxide metabolites

O<sub>2</sub><sup>·-</sup>: superoxide

ONOO<sup>-</sup>: peroxynitrite

RBC: red blood cells

ROS: reactive oxygen species

SCD: sickle cell disease

SCT: sickle cell trait

SOD: superoxide dismutase

T: trained

T<sub>ex</sub>: end of exercise

T<sub>1H</sub>: recovery time at 1 hour

T<sub>2H</sub>: recovery time at 2 hours

T<sub>24H</sub>: recovery time at 24 hours

U: untrained

## INTRODUCTION

Sickle cell disease (SCD) is a hemoglobinopathy resulting from a single mutation in the  $\beta$ -globin chain gene, inducing the substitution of valine for glutamic acid at the sixth amino acid position. This mutation leads to the production of abnormal hemoglobin (HbS). The pathogenesis of sickle cell disease occurs due to the polymerization of deoxygenated hemoglobin S eventually leading to the rigidity and sickling of red blood cells (RBC). The most deleterious pathophysiological effects of sickling include endothelial dysfunction, inflammation, and vaso-occlusion (5). All these events can be traced back to an increase in oxidative stress; defined as a damaging imbalance between the production of oxidants and antioxidants (6, 7). Endothelial dysfunction, which can cause increased adhesion in the vessels leading to vaso-occlusion, hypoxia, and hemolysis, is notably due to the impairment of nitric oxide bioavailability (8, 9). Vaso-occlusion and hypoxia can generate superoxide, while hemolysis can inhibit NO production while generating more reactive oxygen species (6).

Subjects who present both normal (HbA) and sickled hemoglobin (HbS) are identified as sickle cell trait (SCT) carriers. SCT is usually considered to be a benign and asymptomatic condition (10). However, several authors suggest that SCT should be reclassified as a disease state (11, 12). In fact, SCT has been linked as a co-factor for morbidity and mortality (12–14) due to complications at rest (13) and during exercise (1, 15, 16) particularly in hypoxic conditions (1). An increasing number of studies have reported exercise-related sudden deaths in SCT carriers (1, 10, 16, 17). The high incidence of exercise-related deaths in SCT could be a result of RBC abnormalities, such as decreased red blood cell deformability (12, 18) and endothelial damage (13), associated with an increase in oxidative stress (19, 20). This hypothesis is supported by the fact that i) RBC sickling may increase during exercise in SCT

carriers (21), ii) sickle erythrocytes overproduce ROS (19, 20), iii) SCT carriers increase RBC oxidative stress during exercise (22) and iv) ROS induce a cascade of events including endothelial dysfunction and adhesion potentially leading to vascular occlusion (9). Taken together, these results lead to the hypothesis that oxidative stress could be involved in exercise-related complications through the vascular dysfunction mechanism seen in SCT.

In various situations, exercise training has been shown to decrease oxidative stress through an upregulation in the antioxidant system thereby halting the overproduction of oxidants (23–26). In turn, this may improve cardiovascular function by reducing endothelial dysfunction, inflammation, and adhesion (8, 27). A recent paper by Aufradet et al (4) demonstrated that the increased endothelial activation commonly occurring in SCT (28, 29) was attenuated in trained SCT carriers compared to the untrained carriers. This effect on adhesion molecules, which are regulated by nitric oxide and stimulated by ROS (30), suggests that a training effect could be under the control of oxidative stress. However, to our knowledge there are no studies dealing with the effects of regular exercise training on oxidative stress and nitric oxide metabolism in sickle cell trait carriers.

The aim of the present study is to test the hypothesis that regular training improves NO metabolism and decreases oxidative stress, adhesion and endothelial dysfunction in SCT subjects.

## **METHODS**

### *Ethical approval*

The protocol was approved by the local ethics committee of Cameroon and was in accordance with the guidelines set by the Declaration of Helsinki. All of the subjects were volunteers and gave their written informed consent before participating in the study.

### *Subjects*

18 sickle cell trait carriers (SCT;  $34.5 \pm 0.8$  % HbS) and 22 subjects with normal hemoglobin (CON) participated in the study. All subjects were male students attending the University of Yaounde II (Soa, Cameroon). SCT and CON groups were divided into two subgroups based on their fitness level. The 11 CON and 8 SCT who reported no regular physical activity for the two previous years were assigned to untrained (U) sub-groups (U-CON and U-SCT). The 11 CON and 10 SCT who practiced soccer on a regular basis ( $>8$ h/week minimum for several years) were assigned to trained (T) sub-groups (T-CON and T-SCT). The group categorization of trained and untrained was confirmed by fitness level measured by relative maximal aerobic power as described in the results. Subjects completed a medical examination which included height and weight measurements, as well as a venous blood test to detect sickle cell trait and HIV. Exclusion criteria included the presence of a known chronic disease (hypertension, HIV), stroke, or recent malaria episode. No previous sickle cell crisis or other incident relating to hemoglobinopathy was reported in any subject.

### *Exercise Protocol*

All the experiments took place at General Hospital of Yaoundé (Cameroon). The subjects were asked to avoid any strenuous exercise the day before the test. All meals before and after the exercise test were provided at the hospital and water was available ad libitum to insure proper nutrition and hydration. An incremental maximal exercise test was performed on a cycle ergometer (Monark, 818E, Stockholm, Sweden). The test began with a 5-min warm up

at 30 watts (W), followed by a work rate of 70 W which was continuously increased by 35 W every three minutes until volitional exhaustion was reached. Heart rate was collected throughout the exercise using a chest belt monitor (Polar Electro, Kempele, Finland). Maximal heart rate (HR<sub>max</sub>; beats/min) was considered the highest recorded heart rate during the test. Maximal aerobic power (MAP; W) was assessed by linear interpolation from the HR versus work rate curve. Blood samples were drawn from a catheterized antecubital vein on the non-dominant arm and were collected at baseline (Base), immediately at the end of the exercise test (T<sub>ex</sub>) and after 1 hour, 2 hours, and 24 hours of recovery (T<sub>1h</sub>, T<sub>2h</sub>, T<sub>24h</sub>, respectively) in EDTA tubes. The samples were used to measure levels of oxidative stress (AOPP, Protein Carbonyl, MDA, and Nitrotyrosine), antioxidant (catalase, GPX, and SOD), and NO metabolism (NO<sub>x</sub>) markers, and adhesion molecules (P-selectin and E-selectin).

*SCT confirmation,  $\alpha$ -thalassemia, and hematological parameters*

To test for SCT, blood samples were collected in EDTA tubes at rest and the various Hb were isolated and quantified by ion-exchange high performance liquid chromatography (HPLC) (Variant I, Beta Thal Short Program: Bio-Rad Laboratories, Hercules, CA). Positive test results for SCT were determined by the presence of HbS, but only if less than 50% of total Hb. To test for the co-existent of  $\alpha$ -thalassemia, the technique described by Chong and al (31) was used. The only type of  $\alpha$ -thalassemia found in some SCT carriers was the heterozygous form marked by a deletion of 3.7 kb of DNA, containing one of the two linked  $\alpha$ -globin genes ( $\alpha\alpha/\alpha^{3.7}$ ). No other genetic Hb abnormality was found in this population. Blood for the hematological measurements was collected at rest in EDTA tubes and was analyzed using a hematology analyzer (Abbott Cell Dyn 1800 hematology analyzer, Block Scientific, NY, USA).

*Oxidative stress and anti-oxidant assessment*

The blood samples collected at baseline, T<sub>ex</sub>, T<sub>1h</sub>, T<sub>2h</sub> and T<sub>24h</sub> were centrifuged and the aliquoted plasma was stored at -80°C until analysis. All samples were assessed within the same time period.

*Plasma advanced oxidation protein products (AOPP).* AOPP were determined in blood plasma using the semi-automated method developed by Witko-Sarsat et al (32), as previously described (25). AOPP were measured by spectrometry on a microplate reader (TECAN Infinite M200, Männedorf, Switzerland) and were calibrated with a chloramine-T solution that absorbs at 340 nm in the presence of potassium iodide. The absorbance of the reaction was immediately read at 340 nm on the microplate reader against a blank containing 200 µl of PBS. AOPP activity was expressed as micromoles per liter of chloramines-T equivalents.

*Protein Carbonyl.* Measurements of protein carbonyls can be used as an index of oxidative injury. Protein carbonyls were measured by spectrophotometry at 380 nm using 2,4-dinitrophenylhydrazine according to a method of Levine et al (33).

*Catalase.* Catalase activity in the plasma was determined by the method of Johansson and Borg (34), using hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) as a substrate, and formaldehyde as a standard. Catalase activity was determined by the formation rate of formaldehyde induced by the reaction of methanol and H<sub>2</sub>O<sub>2</sub> using catalase as enzyme.

*Glutathione Peroxidase (GPX).* GPX in the plasma was determined by the modified method of Paglia and Valentine (35), using hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) as a substrate. GPX was

determined by the rate of oxidation of NADPH to  $\text{NADP}^+$  after addition of glutathione reductase (GR), reduced glutathione (GSH), and NADPH.

*Malondialdehyde (MDA)*. Although MDA assay shows methodological limitations (36), it is the most common lipid peroxidation marker and it is still widely used as marker of oxidative stress. Concentrations of plasma MDA were determined as thiobarbituric reactive substances by a modified method of Ohkawa et al (37), as previously described (23).

*Nitrite and Nitrate (NOx)*. The end-products of endothelium nitric oxide, nitrites and nitrates, were measured in the plasma using reagents purchased from Sigma-Aldrich, based on methods previously described (38). The sum of nitrite and nitrate in the plasma (NOx) is considered an index of nitric oxide production (19).

*Nitrotyrosine*. Concentrations of plasma nitrotyrosine, as end product of protein nitration by  $\text{ONOO}^-$ , were measured as previously described (39). Nitrotyrosine was measured using reagents purchased from Sigma-Aldrich.

*Superoxide dismutase (SOD)*. The quantitative determination of the SOD activity was performed using the Beauchamps and Fridovich's method (40), slightly modified by Oberley and Spitz (41). SOD activity was determined by the degree of inhibition of the reaction between superoxide radicals, produced by a hypoxanthine-xanthine oxidase system, and nitroblue tetrazolium.



*Adhesion molecule assessment.* sP-selectin and sE-selectin were assessed by ELISA according to the manufacturer's instructions (Diacclone systems, Besançon, France).

### *Statistical Methods*

The results are presented as mean  $\pm$  SD. Anthropometric and hematologic characteristics were compared using a two-way ANOVA with Fisher LSD post-hoc. The data related to oxidative stress markers and antioxidants were compared between groups using a two-way Repeated-Measures ANOVA with Fisher LSD post-hoc. Pearson's coefficient correlations were used to determine the associations between markers of oxidative stress and NOx, nitrotyrosine and adhesion markers, respectively. Statistical significance was determined by a *P* value of less than 0.05. Analyses were conducted using Statistica (version 8.0, Statsoft, Tulsa, OK, USA).

## **RESULTS**

*Anthropometric and hematologic characteristics.* Data for anthropometric, hematologic, and training measurements are detailed in Table 1. Compared to the untrained subjects, the exercise trained group had a significantly higher absolute and relative MAP ( $169 \pm 50$  vs.  $214 \pm 34$  W,  $p = 0.002$  and  $2.78 \pm 0.4$  vs.  $3.17 \pm 0.5$  W/kg,  $p = 0.006$ , for U and T, respectively). Furthermore, there is no differences in absolute or relative MAP among the U and T subgroups i.e., between U-SCT and U-CON (non-significant: NS) and between T-SCT and T-CON (NS). There were no significant differences in %HbS between U-SCT and T-SCT. Whereas no significant differences appeared in age, RBC count, or maximal heart rate among the four groups, U-SCT was taller and U-CON was lighter than the other 3 groups ( $p < 0.05$ ).

T-SCT subjects had a significantly higher platelet count than U-SCT and U-CON (see Table 1).

*Oxidative stress markers at baseline and after maximal exercise* (Table 2).

*AOPP.* AOPP concentrations were not different among the four groups at baseline (NS) but were significantly higher at  $T_{ex}$  than at baseline when the four groups were pooled ( $p < 0.001$ ). Furthermore, AOPP concentrations were significantly higher in U-SCT than in the three other groups (training  $\times$  hemoglobin crossed effect,  $p < 0.05$ ).

**TABLE 1. Anthropometric, Hematologic, and Training Measurements of the study population.**

|                     | Untrained     |               | Trained      |              |
|---------------------|---------------|---------------|--------------|--------------|
|                     | CON           | SCT           | CON          | SCT          |
| %HbS                | N/A           | 36.2 ± 3.1    | N/A          | 33.2 ± 3.2   |
| α-thal              | N/A           | 3/8           | N/A          | 7/10         |
| Age (yr)            | 22.7 ± 1.8    | 23.5 ± 3.0    | 24.6 ± 1.4   | 23.10 ± 2.3  |
| Height (cm)         | 169.2 ± 3.8*  | 178.0 ± 4.4   | 173.7 ± 5.5* | 172.1 ± 3.1* |
| Weight (kg)         | 60.1 ± 5.7    | 69.5 ± 5.5§   | 69.2 ± 4.8§  | 65.6 ± 6.1§  |
| RBC                 | 5.2±0.7       | 5.3±0.5       | 5.1±0.1      | 5.5±0.2      |
| Platelets           | 164.1 ± 81.0¥ | 172.7 ± 82.5¥ | 206.6 ± 95.2 | 266.9 ± 54.7 |
| MAP (W)             | 154.1 ± 16.7¥ | 190.4 ± 11.5¥ | 214.6 ± 11.3 | 212.5 ± 10.4 |
| Max HR(beats/min)   | 185 ± 5       | 183 ± 4       | 174 ± 3      | 189 ± 4      |
| Relative MAP (W/kg) | 2.81 ± 0.36¥  | 2.73 ± 0.35¥  | 3.11 ± 0.53  | 3.24 ± 0.43  |

Values are presented as means ± SD. CON: healthy subjects; SCT: carriers of sickle cell trait; MAP: maximal aerobic power; %HbS: percent of S hemoglobin. \*Significant difference compared to U-SCT ( $p < .05$ ); §Significant difference compared to U-CON ( $p < .05$ ); ¥Significant difference compared to trained subjects (CON and SCT,  $p < .05$ ).

*MDA.* At baseline, trained subjects had higher levels of MDA than untrained subjects ( $p = 0.014$ ) whereas there were no differences between SCT carriers and CON subjects. MDA concentrations were significantly increased at  $T_{\text{ex}}$ ,  $T_{1\text{h}}$ , and  $T_{2\text{h}}$  compared to baseline in U-SCT subjects, while they were decreased at  $T_{1\text{h}}$ ,  $T_{2\text{h}}$  and  $T_{24\text{h}}$  in trained subjects (Table 2). The percentage increase from baseline was significantly higher in U-SCT than the 3 other groups at  $T_{\text{ex}}$ ,  $T_{1\text{h}}$ ,  $T_{2\text{h}}$ , and  $T_{24\text{h}}$  (Figure 1).

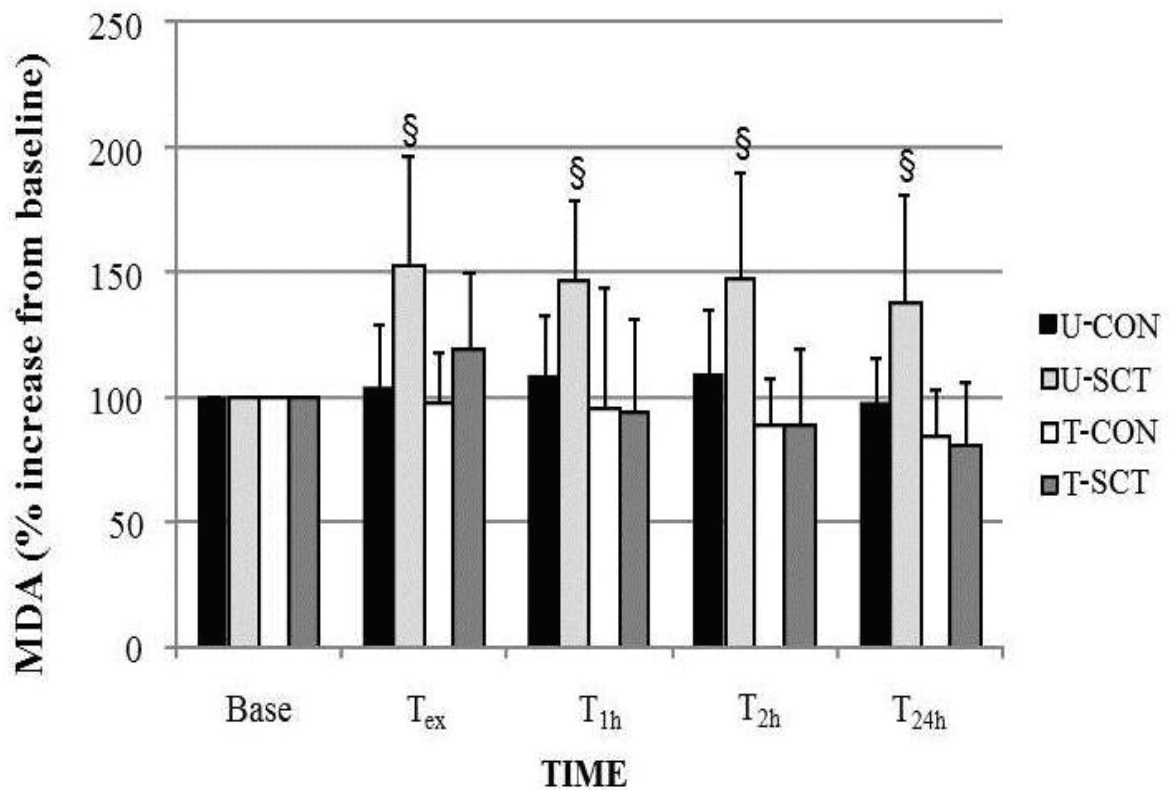


Figure 1: Effect of a maximal exercise test on the plasma concentrations of MDA in trained (T-SCT and T-CON) and untrained (U-SCT and U-CON) subjects as a percent increase from baseline (Base), at the end of exercise (T<sub>ex</sub>), and during recovery (T<sub>1h</sub>, T<sub>2h</sub>, T<sub>24h</sub>). Values are presented as mean  $\pm$  SD. §Significant difference from other 3 groups ( $p < .05$ ).

*Protein Carbonyl.* There was no difference in protein carbonyl content among groups at baseline (NS). Protein carbonyl was significantly higher in all four groups at T<sub>ex</sub> compared to baseline (U-CON: +117 %,  $p = 0.0002$ ; U-SCT: +124 %,  $p < 0.0001$ ; T-CON: +77 %,  $p < 0.0001$ ; T-SCT: +71 %,  $p = 0.0005$ ). U-SCT protein carbonyl concentrations were significantly higher than in the other three groups at T<sub>ex</sub> ( $p = 0.003$  vs. T-CON;  $p = 0.036$  vs.

U-CON; and  $p = 0.005$  vs. T-SCT). Protein carbonyl levels at  $T_{1h}$ ,  $T_{2h}$ , and  $T_{24h}$  were not significantly different from baseline values regardless of the group.

*Nitrotyrosine.* Nitrotyrosine levels were not different at baseline among the four groups (NS). An overall training effect (ANOVA trained vs. untrained independently to time point) was observed with higher nitrotyrosine concentrations in untrained subjects compared to their trained counterparts ( $p = 0.01$ ). U-SCT was the only group with a significant increase in nitrotyrosine at  $T_{ex}$  compared to Base ( $p = 0.001$ ). Thus, U-SCT had higher nitrotyrosine concentrations at  $T_{ex}$  than the other three groups ( $p = 0.048$  vs. U-CON;  $p < 0.0001$  vs. T-CON; and  $p = 0.003$  vs. T-SCT). At  $T_{1h}$ , U-CON, U-SCT, and T-CON exhibited higher nitrotyrosine concentrations than at baseline.

**TABLE 2. Plasma AOPP, MDA, nitrotyrosine, and protein carbonyl at Base, immediately after a maximal exercise test ( $T_{ex}$ ), and during the recovery ( $T_{1h}$ ,  $T_{2h}$ ,  $T_{24h}$ ) in U-CON, U-SCT, T-CON, and T-SCT.**

|                               |           | Untrained        |                   | Trained          |                  | Time Effect |
|-------------------------------|-----------|------------------|-------------------|------------------|------------------|-------------|
|                               |           | CON              | SCT               | CON              | SCT              |             |
| AOPP<br>( $\mu\text{mol/L}$ ) | Base      | 61.2 $\pm$ 24.7  | 78.7 $\pm$ 53.6   | 80.9 $\pm$ 48.5  | 64.4 $\pm$ 16.5  |             |
|                               | $T_{ex}$  | 127.7 $\pm$ 49.6 | 214.0 $\pm$ 136.9 | 110.4 $\pm$ 66.9 | 123.7 $\pm$ 65.4 | *           |
|                               | $T_{1h}$  | 73.4 $\pm$ 59.3  | 125.8 $\pm$ 81.6  | 76.9 $\pm$ 54.5  | 57.4 $\pm$ 49.4  |             |
|                               | $T_{2h}$  | 99.5 $\pm$ 87.6  | 136.5 $\pm$ 87.3  | 69.9 $\pm$ 54.1  | 88.3 $\pm$ 64.1  |             |
|                               | $T_{24h}$ | 86.0 $\pm$ 30.7  | 124.2 $\pm$ 96.6  | 131.3 $\pm$ 75.5 | 125.6 $\pm$ 69.2 | *           |
| Hb x Training Effect          |           | §                |                   |                  |                  |             |

|                               |                  |                           |                    |                           |                           |     |
|-------------------------------|------------------|---------------------------|--------------------|---------------------------|---------------------------|-----|
| MDA<br>( $\mu\text{mol/L}$ )  | Base             | 23.4 $\pm$ 2.0            | 20.5 $\pm$ 6.2     | 27.4 $\pm$ 6.2 $\dagger$  | 27.3 $\pm$ 6.5 $\dagger$  |     |
|                               | T <sub>ex</sub>  | 24.1 $\pm$ 5.5            | 32.1 $\pm$ 9.3*    | 27.2 $\pm$ 6.6            | 31.3 $\pm$ 5.2            |     |
|                               | T <sub>1h</sub>  | 25.2 $\pm$ 4.4            | 29.4 $\pm$ 5.1*    | 19.8 $\pm$ 5.6*£          | 23.7 $\pm$ 5.2*£          |     |
|                               | T <sub>2h</sub>  | 25.4 $\pm$ 5.4            | 28.1 $\pm$ 6.5*    | 23.0 $\pm$ 4.1*£          | 22.7 $\pm$ 4.3*£          |     |
|                               | T <sub>24h</sub> | 22.7 $\pm$ 2.0            | 26.4 $\pm$ 5.2     | 23.2 $\pm$ 3.8*£          | 21.3 $\pm$ 5.9*£          |     |
| Protein Carbonyl<br>(nmol/mL) | Base             | 50.5 $\pm$ 12.0           | 49.2 $\pm$ 11.4    | 46.8 $\pm$ 14.4           | 51.8 $\pm$ 10.3           |     |
|                               | T <sub>ex</sub>  | 89.5 $\pm$ 12.0 $\dagger$ | 111.4 $\pm$ 36.6   | 81.6 $\pm$ 16.0 $\dagger$ | 84.8 $\pm$ 25.0 $\dagger$ | *   |
|                               | T <sub>1h</sub>  | 40.8 $\pm$ 21.9           | 53.6 $\pm$ 21.3    | 62.6 $\pm$ 19.3           | 52.8 $\pm$ 15.8           |     |
|                               | T <sub>2h</sub>  | 36.3 $\pm$ 6.9            | 43.5 $\pm$ 9.6     | 52.5 $\pm$ 15.6           | 39.8 $\pm$ 11.6           |     |
|                               | T <sub>24h</sub> | 37.1 $\pm$ 7.4            | 46.7 $\pm$ 18.4    | 46.2 $\pm$ 13.8           | 41.8 $\pm$ 10.3           |     |
| Nitrotyrosine<br>(nmol/L)     | Base             | 82.8 $\pm$ 28.7           | 67.6 $\pm$ 20.8    | 65.4 $\pm$ 21.3           | 95.2 $\pm$ 29.4           |     |
|                               | T <sub>ex</sub>  | 94.3 $\pm$ 38.2           | 132.7 $\pm$ 27.7§* | 97.1 $\pm$ 39.1           | 75.1 $\pm$ 51.2           |     |
|                               | T <sub>1h</sub>  | 130.5 $\pm$ 22.3*£        | 115.6 $\pm$ 32.9*  | 104.9 $\pm$ 24.4*         | 92.0 $\pm$ 27.0           |     |
|                               | T <sub>2h</sub>  | 102.1 $\pm$ 27.5          | 95.6 $\pm$ 43.4    | 97.3 $\pm$ 34.3           | 80.0 $\pm$ 30.1           |     |
|                               | T <sub>24h</sub> | 76.7 $\pm$ 25.1           | 83.4 $\pm$ 37.0    | 47.0 $\pm$ 29.7           | 52.0 $\pm$ 29.8           | £ # |
| Training Effect               |                  |                           |                    | ¥                         | ¥                         |     |

Values are presented as means  $\pm$  SD. CON: healthy subjects; SCT: carriers of sickle cell trait; AOPP: advanced oxidation protein products; MDA: malondialdehyde. \*Significant difference compared to BASE; §Significant difference compared to the other times; £ Significant difference compared to Tex. # Significant difference compared to T1h and T2h; § Significant difference compared to the three other groups;  $\dagger$ Significant difference compared to U-SCT ( $p < .05$ ); ¥ Significant difference compared to untrained subjects ( $p < .05$ ).

*Antioxidant markers at baseline and after maximal exercise (See Table 3).*

*SOD*. No significant inter-group difference was observed in baseline SOD activity. Compared to baseline, a significant increase of SOD activity was observed at  $T_{ex}$  ( $p < 0.0001$ ) and  $T_{1h}$  ( $p = 0.002$ ) in trained subjects, whereas no significant variations were observed in their untrained counterparts. Furthermore, SOD activity was significantly higher in the trained subjects than in the untrained ones at  $T_{ex}$  ( $p = 0.015$ , Figure 2).

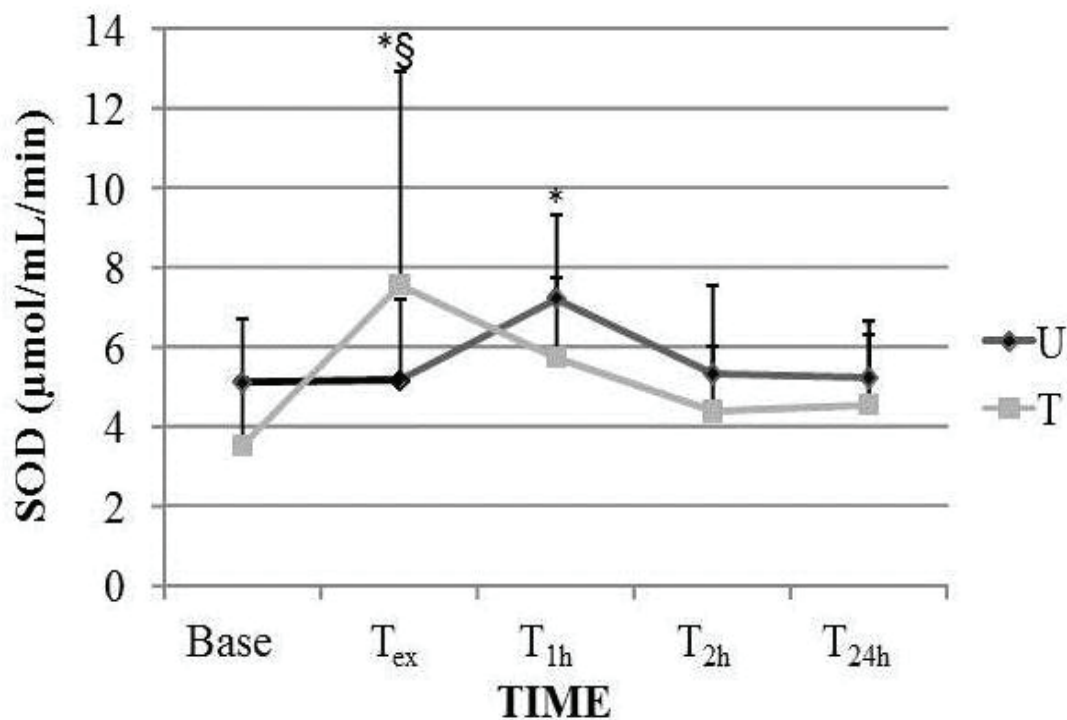


Figure 2: Effects of a maximal exercise test on the plasma activity of SOD in untrained (U) and trained (T) subjects at baseline (Base), at the end of exercise ( $T_{ex}$ ), and during recovery ( $T_{1h}$ ,  $T_{2h}$ , and  $T_{24h}$ ). Values represented as means  $\pm$  SD. \*Significant difference between EX and Base ( $p < .003$ ); §Significant difference between U and T at  $T_{ex}$  ( $p = .016$ ).

*Catalase*. A training effect was observed at baseline with significantly higher catalase activities in trained subjects than their untrained counterparts ( $p < 0.001$ ). Trained subjects

expressed lower activities of catalase at  $T_{\text{ex}}$  ( $p < 0.001$ ),  $T_{2\text{h}}$  ( $p < 0.001$ ) and  $T_{24\text{h}}$  ( $p < 0.001$ ) than at baseline.

*GPX.* No significant inter-group differences were observed at baseline for GPX activity. Regardless of the training or hemoglobin status, GPX was significantly higher at  $T_{\text{ex}}$ ,  $T_{1\text{h}}$  and  $T_{2\text{h}}$  compared to baseline (baseline:  $46.3 \pm 18.8$  vs.  $T_{\text{ex}}$ :  $111.6 \pm 31.7$ ,  $p < 0.0001$ , vs.  $T_{1\text{h}}$ :  $102.9 \pm 21.6$   $p < 0.0001$ , and vs.  $T_{2\text{h}}$ :  $77.9 \pm 21.6$ ,  $p < 0.0001$ ; 4 groups pooled). At  $T_{1\text{h}}$  GPX was significantly higher in the trained subjects than in their untrained counterparts ( $112.0 \pm 14.7$  vs.  $87.7 \pm 9.5$ , respectively,  $p = 0.041$ ).

**TABLE 3. Plasma anti-oxidant enzyme activities and NOx at Base, immediately after a maximal exercise test ( $T_{\text{ex}}$ ), and during recovery ( $T_{1\text{h}}$ ,  $T_{2\text{h}}$ ,  $T_{24\text{h}}$ ) in U-CON, U-SCT, T-CON, and T-SCT.**

|                                        |                  | Untrained     |               | Trained        |                | Time x training effect |
|----------------------------------------|------------------|---------------|---------------|----------------|----------------|------------------------|
|                                        |                  | CON           | SCT           | CON            | SCT            |                        |
| SOD<br>( $\mu\text{mol/mL/min}$ )      | Base             | 5.6 $\pm$ 2.0 | 5.0 $\pm$ 1.2 | 3.5 $\pm$ 2.3  | 3.4 $\pm$ 1.4  |                        |
|                                        | $T_{\text{ex}}$  | 6.0 $\pm$ 1.3 | 4.3 $\pm$ 2.5 | 6.5 $\pm$ 2.7* | 8.7 $\pm$ 7.7* | †                      |
|                                        | $T_{1\text{h}}$  | 5.7 $\pm$ 1.4 | 7.8 $\pm$ 1.2 | 5.9 $\pm$ 1.9* | 6.3 $\pm$ 1.4* |                        |
|                                        | $T_{2\text{h}}$  | 5.8 $\pm$ 3.4 | 5.3 $\pm$ 1.9 | 4.8 $\pm$ 1.9  | 4.2 $\pm$ 1.4  |                        |
|                                        | $T_{24\text{h}}$ | 5.1 $\pm$ 1.5 | 5.0 $\pm$ 1.8 | 5.0 $\pm$ 2.0  | 4.0 $\pm$ 1.7  |                        |
| Catalase<br>( $\mu\text{mol/mL/min}$ ) | Base             | 3.8 $\pm$ 1.0 | 4.0 $\pm$ 1.8 | 5.0 $\pm$ 0.9  | 6.1 $\pm$ 1.6  | †                      |
|                                        | $T_{\text{ex}}$  | 3.4 $\pm$ 0.8 | 4.3 $\pm$ 1.5 | 3.5 $\pm$ 1.6* | 4.4 $\pm$ 1.2* |                        |
|                                        | $T_{1\text{h}}$  | 4.6 $\pm$ 0.8 | 4.3 $\pm$ 1.2 | 4.6 $\pm$ 1.7  | 5.1 $\pm$ 1.1  |                        |
|                                        | $T_{2\text{h}}$  | 4.1 $\pm$ 1.1 | 3.7 $\pm$ 1.1 | 3.9 $\pm$ 1.0* | 3.7 $\pm$ 1.2* |                        |
|                                        | $T_{24\text{h}}$ | 3.6 $\pm$ 0.6 | 3.5 $\pm$ 0.6 | 3.5 $\pm$ 0.8* | 3.8 $\pm$ 0.5* |                        |



|                                   |                  |                   |                   |                  |                   |   |
|-----------------------------------|------------------|-------------------|-------------------|------------------|-------------------|---|
| GPX<br>( $\mu\text{mol/mL/min}$ ) | Base             | 28.1 $\pm$ 2.6    | 37.8 $\pm$ 19.8   | 54.9 $\pm$ 25.6  | 54.0 $\pm$ 20.7   |   |
|                                   | T <sub>ex</sub>  | 117.5 $\pm$ 31.6* | 100.0 $\pm$ 36.2* | 126.5 $\pm$ 8.4* | 104.4 $\pm$ 38.2* |   |
|                                   | T <sub>1h</sub>  | 92.3 $\pm$ 7.9*   | 83.1 $\pm$ 16.1*  | 122.9 $\pm$ 9.2* | 104.8 $\pm$ 19.7* | † |
|                                   | T <sub>2h</sub>  | 82.0 $\pm$ 22.1*  | 68.5 $\pm$ 19.0*  | 88.8 $\pm$ 26.0* | 73.2 $\pm$ 17.2*  |   |
|                                   | T <sub>24h</sub> | 54.9 $\pm$ 32.5   | 60.3 $\pm$ 11.3   | 29.3 $\pm$ 2.9   | 54.9 $\pm$ 24.0   |   |
| NOx<br>( $\mu\text{mol.L}^{-1}$ ) | Base             | 20.5 $\pm$ 2.9    | 19.7 $\pm$ 6.9    | 22.0 $\pm$ 5.9   | 19.8 $\pm$ 3.1    |   |
|                                   | T <sub>ex</sub>  | 20.6 $\pm$ 3.6    | 14.6 $\pm$ 7.0    | 26.6 $\pm$ 8.0*  | 28.8 $\pm$ 11.4*  | † |
|                                   | T <sub>1h</sub>  | 20.9 $\pm$ 6.2    | 22.8 $\pm$ 18.0   | 18.5 $\pm$ 9.1   | 21.5 $\pm$ 9.3    |   |
|                                   | T <sub>2h</sub>  | 24.0 $\pm$ 10.7   | 36.1 $\pm$ 14.5*¥ | 21.2 $\pm$ 7.7   | 19.7 $\pm$ 7.6    |   |
|                                   | T <sub>24h</sub> | 20.8 $\pm$ 7.6    | 20.5 $\pm$ 10.0   | 21.5 $\pm$ 9.1   | 19.9 $\pm$ 6.7    |   |

Values are presented as means  $\pm$  SD. CON: healthy subjects; SCT: carriers of sickle cell trait; SOD: superoxide dismutase; NOx: nitric oxide metabolites; GPX: glutathione peroxidase.\*Significant difference compared to BASE ( $p < .05$ ); †Significant difference compared to Untrained ( $p < .05$ ); ¥Significant difference compared to the three other groups ( $p < .05$ ).

*NOx.* No significant inter-group difference was observed in baseline NOx. NOx was significantly higher in trained subjects, whatever their hemoglobin status (i.e. T-SCT or T-CON) at T<sub>ex</sub> compared to baseline ( $p = 0.037$ ), while NOx remained unchanged in untrained subjects. Consequently, trained subjects had significantly higher NOx values than untrained subjects at T<sub>ex</sub> ( $p = 0.001$ ). Moreover, NOx in U-SCT significantly increased at T<sub>2h</sub> compared to baseline ( $p = 0.0005$ ) and was significantly higher than the 3 other groups ( $p = 0.017$  vs. U-CON;  $p = 0.002$  vs. T-CON, and  $p = 0.006$  vs. T-SCT).

#### *Adhesion Molecules.*

Complete data of adhesion molecules are presented in Aufradet et al (4) (previously published). Briefly, basal plasma concentrations of sP-selectin and sE-selectin were not statistically different among the four groups. Plasma sE-selectin significantly increased in all

groups at the end of the exercise as compared with baseline levels and returned to baseline value 1 hour after the end of exercise ( $T_{1h}$ ). Although incremental exercise did not statistically modulate sP-selectin concentrations in T subjects, a significant increase in these concentrations was measured in their untrained counterparts between Base and  $T_{ex}$ . These concentrations returned to basal values 1 hour after the end of exercise.

**Table 4: Correlations between oxidative stress and adhesion markers (pooled subjects).**

| Variables          | Timepoint             | Pearson's Correlation | P value |
|--------------------|-----------------------|-----------------------|---------|
| NOx with           |                       |                       |         |
| AOPP               | Baseline              | -.6988                | .000    |
| GPX                | Baseline vs. $T_{1h}$ | .4093                 | .031    |
| MDA                | Baseline vs. Ex       | -.3968                | .036    |
| AOPP               | Baseline vs. $T_{1h}$ | -.4762                | .007    |
| Nitrotyrosine with |                       |                       |         |
| Catalase           | Exercise              | -.5694                | .002    |
| Carbonyl           | Baseline vs. Ex       | .5787                 | .002    |
| AOPP               | Baseline vs. $T_{2h}$ | .5671                 | .004    |
| E-selectin with    |                       |                       |         |
| Catalase           | Exercise              | -.3977                | .020    |
| MDA                | Baseline vs. $T_{1h}$ | .3615                 | .050    |
| P-selectin with    |                       |                       |         |
| AOPP               | Exercise              | .4274                 | .008    |

|          |                 |       |      |
|----------|-----------------|-------|------|
| Carbonyl | T <sub>2h</sub> | .3459 | .039 |
| AOPP     | Baseline vs. Ex | .3591 | .029 |

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Values are presented as Pearson's correlation coefficients and corresponding p values. NOx: nitric oxide metabolites; AOPP: advanced oxidative protein product; MDA: malondialdehyde; GPX: glutathione peroxidase.

### *Correlations.*

Significant correlations were observed between markers of oxidative stress, nitric oxide, and markers of adhesion. The correlations on the pooled subjects are presented in Table 4. We found also significant relationships between changes (Base vs. T<sub>ex</sub>, T<sub>1h</sub> or T<sub>2h</sub>) in oxidative stress and sE- and sP-selectins or NO metabolism (Table 4). Finally, percentage of HbS was negatively correlated with the NOx increase between baseline and T<sub>ex</sub> ( $r = -0.59$ ,  $p = 0.021$ ) i.e., the more the HbS content, the lower the increase in NOx in response to exercise.

## **DISCUSSION**

The aim of this study was to investigate the impact of regular physical activity on plasma markers of oxidative stress in SCT carriers. In support of our hypotheses, the results of the present study demonstrated that regular physical activity i) blunted the increase in oxidative stress and the decrease in NO metabolism observed in SCT and ii) up-regulated the antioxidant enzymes activities (SOD and GPX) in response to exercise in SCT. In addition, we reported a strong association between changes in various oxidative stress markers in response to exercise and the corresponding changes in sP- and sE-selectins. Although no

causality can be assumed, these correlations strengthen the hypothesis that ROS may be involved in endothelial adhesion in HbS carriers.

*General considerations on the effects of hemoglobin and training status on hematologic and training measurements.*

Maximal aerobic power was similar between SCT and control subjects, as supported by previous studies. Conversely, MAP was higher in the trained subjects compared to the untrained subjects. In agreement with several authors (42, 43), we found that trained subjects had a higher platelet count than untrained subjects. The T-SCT subjects had non-significantly higher platelet counts than the other three groups. Although an increased platelet count is a risk factor for vaso-occlusive crises in SCD (44), this increase was not significant and is probably due to a training effect rather than a hemoglobin effect.

*General considerations on the effects of maximal exercise and training on oxidative stress and antioxidant markers in healthy subjects.*

Regarding the results obtained in CON, the present study is in complete agreement with data reported in the literature. First, the overall increase in various markers of oxidative stress observed in response to the maximal exercise test is consistent with the results of previous studies (45, 46). Second, strong evidence indicates that exercise training can have a beneficial effect on oxidative stress (26, 45, 47). As supported by Miyazaki et al (48), trained subjects have an improved ability to endure the stress of a maximal exercise bout. A lower level of oxidative stress was also evident in the trained subjects of our study. In fact, T-CON subjects had a reduced increase in all markers of oxidative stress in response to the maximal exercise test compared to their untrained counterparts.

It has been reliably shown that individuals partaking in exercise training have higher levels of antioxidant enzymes and exhibit greater protection against exercise-induced oxidative stress (49, 50). The reactive oxygen species produced during exercise training can result in a stimulation of antioxidant defense (51, 52). The exercise trained subjects in our study produced a significant increase in SOD and GPX, which was accompanied by lower oxidative stress. This decrease in oxidative stress likely occurs due to the improved antioxidant adaptation. Our results are in agreement with several other studies that found an improved antioxidant response relating to exercise training (23, 53).

Our study shows also higher baseline levels of MDA in our trained subjects (47, 54). The high level of resting MDA in trained subjects could be related to the ability of lipid peroxidation products to regulate and modulate cell signaling and gene expression (55). Oxidized lipids can interact with receptors that are known to activate antioxidant enzymes such as catalase and SOD (56), and can improve cellular tolerance against pro-oxidant attacks (55). It is therefore possible that higher levels of lipid peroxidation products, such as MDA, are needed to maintain antioxidant status, such as we saw for catalase and GPX (even if non-significant).

#### *Exercise training blunts oxidative stress in SCT carriers*

HbS, which represents around 80% of total hemoglobin in SCD patients, can generate a 2-fold greater quantity of reactive oxygen species than HbA (19, 57). In sickle cell trait carriers, in whom HbS represents around 40% of total hemoglobin, we found that oxidative stress varied little at baseline from healthy subjects. This is in agreement with other studies that found no difference between SCT carriers and healthy subjects at baseline (4, 22, 58). However, in response to acute exercise, SCT carriers can experience higher oxidative stress (3, 22). Our study reinforces this idea as it shows that U-SCT had a significantly greater increase in MDA

and AOPP in response to a maximal exercise bout than the healthy subjects. The increased morbidity or mortality in SCT could be attributed to higher levels of oxidative stress(12).

However, the main finding of the present study is that the well-known benefits of exercise training in healthy subjects are well translated in the SCT subjects. In response to the maximal exercise test, T-SCT subjects i) exhibited lower AOPP, MDA, nitrotyrosine, and protein carbonyl levels than U-SCT, and ii) responded similarly to the T-CON subjects with regards to the protein carbonyl, MDA, and AOPP. In addition to the fact that these results mimic those already seen in many other disease, such as cardiovascular disease (59), diabetes (60), and menopause (23), they emphasize the beneficial effects of exercise training on oxidative stress in SCT carriers.

*Antioxidant defense system is improved in exercise trained SCT carriers.*

The improvement in the antioxidants defense system seen in exercise training is due to a ROS-generated stimulation triggering antioxidant enzyme activation (51, 52). The maximal exercise test which normally increases oxidative stress was met by a concomitant increase in antioxidant enzyme defenses, in all but the U-SCT carriers. Repeated habitual exercise can cause antioxidants to increase in response to the repeated oxidative stress. This has been supported *in vitro* where the treatment of pro-oxidants stimulated a significant increase in antioxidants (61). Interestingly, this did not occur in U-SCT subjects, as they had a delayed response of SOD and a reversed response of catalase. This supports studies which showed levels of SOD and catalase decrease in proportion to disease severity (20, 62). The response to SOD and catalase could indicate an impaired antioxidant status, meaning a reduced ability to buffer the excess oxidative stress.

The trait by itself can result in an increase in oxidative stress and impairment in NO, and when paired with an acute stress such as a maximal exercise test, this response is augmented; as observed with U-SCT. However, exercise training is able to stimulate antioxidants in order to respond to the overload of oxidative stress. This improved response to oxidative stress allows SCT carriers to respond similarly to CON subjects, as demonstrated by our study. This indicates that, as far as oxidative stress is concerned, training can override the negative consequence of SCT, rendering these subjects as controls.

### *Nitric Oxide*

Nitric oxide, an important mediator of vasodilation, has been shown to be amplified in response to exercise training (45, 47, 63, 64). The present study supports these findings, as the SCT and CON trained subjects significantly increased NOx immediately after the exercise test, while the untrained subjects slightly decreased. Our study suggests that exercise training may inhibit NO degradation through an upregulation of antioxidants and a decrease in ROS. We found that the percent change (%Δ) of NOx levels were positively correlated with GPX levels at T<sub>1h</sub>, and were negatively correlated with MDA levels (%Δ) at T<sub>ex</sub> and AOPP levels (%Δ) at T<sub>1h</sub>. Nitrotyrosine, which represents the nitration activity of peroxynitrite (ONOO<sup>-</sup>) produced through the reaction of NO and superoxide anion (O<sub>2</sub><sup>-</sup>), tended to increase in the untrained subjects compared to the trained subjects (p = 0.055) and was significantly higher in the U-SCT carriers compared to T-SCT carriers (p = 0.003). This is in accordance with other studies which found a decrease in nitrotyrosine levels in exercise trained subjects (23, 65). Although our correlations do not suggest causality, these results i) could indicate a reduced O<sub>2</sub><sup>-</sup> production and ii) suggest an increase in NO bioavailability as a result of reduced oxidative stress (23, 47) in trained SCT carriers than in their untrained counterparts.

The improvement in NOx levels in response to physical activity level is reflected in the SCT subjects as well. T-SCT subjects had a similar improvement in NOx response as the T-CON subjects did. Interestingly, the U-SCT had a delayed peak in NOx at T<sub>2h</sub>, which was associated with an elevation in oxidative stress. These results emphasize the overall impairment in response to an exhaustive exercise bout. An impaired NO bioavailability is associated with an increase in ROS, hemolysis (66), eNOS uncoupling due to hemolysis-induced arginase, and cell adhesion (8). Because NO has potent anti-adhesive properties which down-regulate adhesion molecule expression maintaining proper endothelial cell function and vasodilation, a NO impairment can increase cell adhesion (8). This is supported by Aufradet et al (4), who found a significant increase in VCAM-1 levels in untrained SCT subjects immediately after exercise (U-SCT: 1738 ± 98 ng/ml vs. T-SCT: 1248 ± 131 ng/ml; p<.05); the exact same time point as the decreased NOx response in U-SCT carriers. As ROS markers were positively correlated with sP- and sE-selectins and negatively correlated with antioxidant markers, these data suggest that a NO-induced increase in oxidative stress could exacerbate endothelial and/or platelets activation. However, it should be noted that there were no differences between sP-selectin and sE-selectin in the different subjects. Therefore, the training-induced decrease in oxidative stress and increase in NO bioavailability could be favorable for the health of SCT carriers involved in regular exercise by dampening risk of sickling, morbidity, and mortality.

This study suggests that exercise training can improve the response to oxidative stress in SCT carriers. However, at this time, most studies involving different intensities and durations of exercise have found conflicting results in relation to coagulation activity, RBC deformability, inflammation, and adhesion in SCT (see review by Connes et al (2)). However, these events may be compounded by other factors such as heat stress, dehydration, and poor physical



conditioning. Baskurt and Meiselman (67), suggested that exercise utilizes the vascular autoregulatory reserve to maintain homeostasis, yet even minor vascular and hemorheologic perturbations in SCT carriers may be augmented in response to exercise.

#### Conclusion.

In conclusion, we found that although there is relatively little difference between sickle cell trait carriers and healthy subjects at rest, a maximal exercise test can inundate the oxidative stress response in U-SCT subjects. We also found that training can reduce the oxidative stress in response to exercise of SCT carriers. Training improves antioxidant and NO availability which can thereafter regulate ROS production. These effects could result in decreased endothelial activation. This study does not allow us to make definitive conclusions about the direct causality between exercise training and oxidative stress and NO improvements. Further information could be concluded using longitudinal studies which focus on mechanistic pathways and more directly evaluate endothelial dysfunction using methods such as flow mediated dilation.

Finally, we believe that the beneficial effects seen in SCT carriers in this study could translate well in SCD patients as well. Although there are large clinical differences between SCT and SCD, both are overwhelmed by an increase in oxidative stress. Because we have shown that exercise training can decrease oxidative stress and improve antioxidant and nitric oxide responses, we believe that the complications known in SCD, such as vaso-occlusion crisis, that are linked to oxidative stress (68, 69) could potentially be reduced. Therefore, an adapted exercise training program could be a relevant option to control the cardiovascular complications of this pathology.

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# **Chapter V**

## **Atherosclerosis & the role of Oxidative Stress and Inflammation**

## Chapter V Atherosclerosis

Atherosclerosis is the leading cause of death in developed nations and will soon likely attain this status worldwide. Atherosclerosis is no longer thought of as a disease ridden by fatty deposits on artery walls. It is a complex cardiovascular disease that involves multiple factors including endothelial dysfunction, oxidized low-density lipoproteins, inflammation, and oxidative stress. The complex mechanisms of atherosclerosis are worsened by certain factors such as diabetes, obesity, dyslipidproteinemia, hypertension, smoking, and inactivity.

The first part of this chapter will illustrate the pathogenesis of plaque development leading to the progression of advanced disease and vulnerability. This will lead into the second discussion on the underlying mechanisms towards plaque disruptions in the context of oxidative stress and inflammation. The third section will focus on non-invasive methods used to investigate plaque vulnerability. Following this section, will be a brief overview of the animal models used to study advanced lesions. Finally, this chapter will conclude with a section on intervention studies, focusing on the interplay of exercise and diet.

### V.1 Plaque Development

Atherosclerosis is a progressive disease beginning in childhood with clinical manifestations occurring during middle and late adulthood. The disease first appears in the aorta, expanding to the coronary arteries in the second decade of life, and finally in the cerebral arteries by the third decade. Lesions tend to develop at branch points, such as at the carotid arteries bifurcation, and at the curvature of smaller vessels, such as in the aortic arch, due to normal hemodynamic stress-induced adaptive intimal thickening (*1*).

### V.1a Vascular Components: The vessel wall.

The arterial wall consists of three separate layers: the intima, media, and adventitia (Figure 13). Each layer is divided by an elastic lamina; an internal elastic lamina between the intima and media, and an external elastic lamina between the media and adventitia. The innermost layer, the intima, consists of a single layer of endothelial cells on the luminal side and a sheet of elastic fibers on the peripheral side. A healthy intima is a very thin layer comprised of an extracellular connective tissue matrix, consisting of mostly proteoglycans and collagen. The middle layer, the media, consists of smooth muscle cells (SMC) held together by the elastic fibers and collagen comprising the extracellular matrix. The outermost layer, the adventitia, consists of a loose matrix of elastin and collagen interspersed with fibroblasts and SMCs (2, 3).

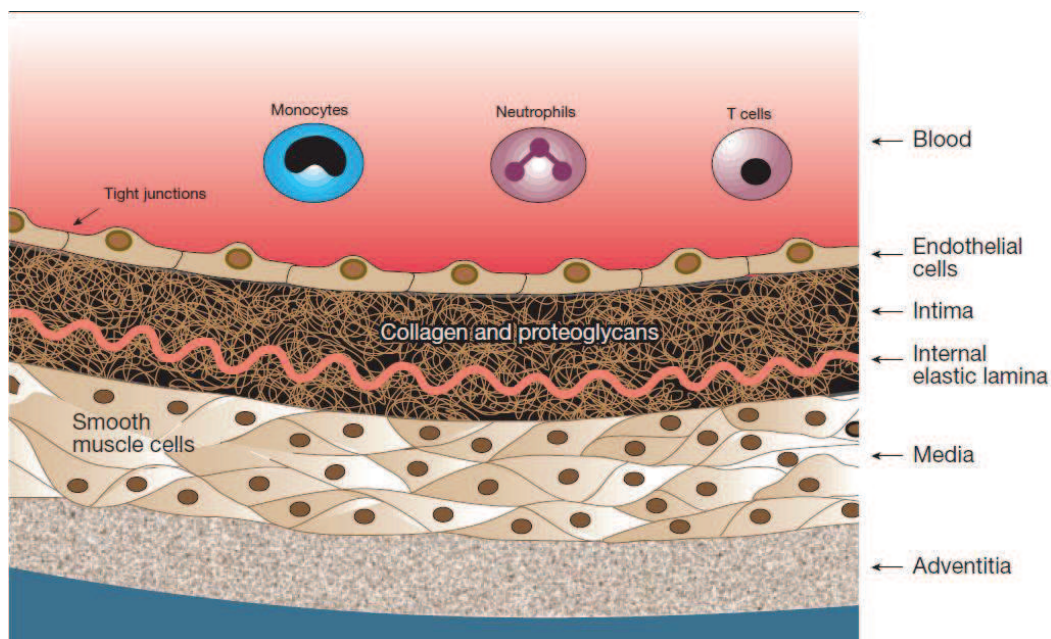


Figure 13: Major layers composing a large artery. The intima is a thin layer consisting of extracellular connective tissue matrix. The media consists of smooth muscle cells. The adventitia consists of connective tissue interspersed with fibroblasts and smooth muscle cells (2).

### **V.1b Vascular Components: The endothelium**

The endothelium is composed of a thin layer of cells which lines the interior surface of blood vessels. Its intercellular tight junction complexes act as a selective barrier between the vessel lumen and the surrounding tissue. The endothelium can generate effector molecules that regulate thrombosis, inflammation, and vascular tone and remodeling (2). Endothelial cells (EC) located in tubular regions where the blood flow is uniform and laminar have ellipsoidal shapes and are aligned to the direction of flow. Those in branching or curving regions where the flow is disturbed have polygonal shapes and no specific orientation. The cells located in these areas have increased permeability to macromolecules and are often the preferred site for lesion formation (4). An excessive or prolonged increase in permeability may lead to inflammation and endothelial dysfunction. Endothelial dysfunction is due to a decrease in nitric oxide, as well as an increase in reactive oxygen species (5).

### **V.2 Pathogenesis of Atherosclerosis.**

The primary initiating event in atherosclerosis is the subendothelial accumulation of LDL. The rate of accumulation is dependent on the levels of circulating LDLs. Once retained in the vessel walls, LDL undergoes modification, such as oxidation, proteolysis, lipolysis, and aggregation, due to exposure to vascular cell oxidative waste. LDL modification contributes to inflammation by stimulating endothelial cells to produce adhesion molecules and growth factors, and smooth muscle cells to secrete chemokines and chemoattractants. The combined action of these secretions attracts monocytes, lymphocytes, mast cells, and neutrophils into the arterial wall. LDL can be highly oxidized by ROS generated by endothelial cells and macrophages (Figure 14). At this stage, the highly oxidized LDLs are engulfed by macrophages, becoming foam cells. As these foam cells accumulate and more inflammatory

cells are activated, necrotic debris and smooth muscle cells accumulate, further provoking inflammation. Fibrous caps develop over the lipid-rich necrotic core, just under the endothelium. As advancing atheroma develops, the fibrous cap can thin and weakened, becoming more susceptible to rupture (2, 6).

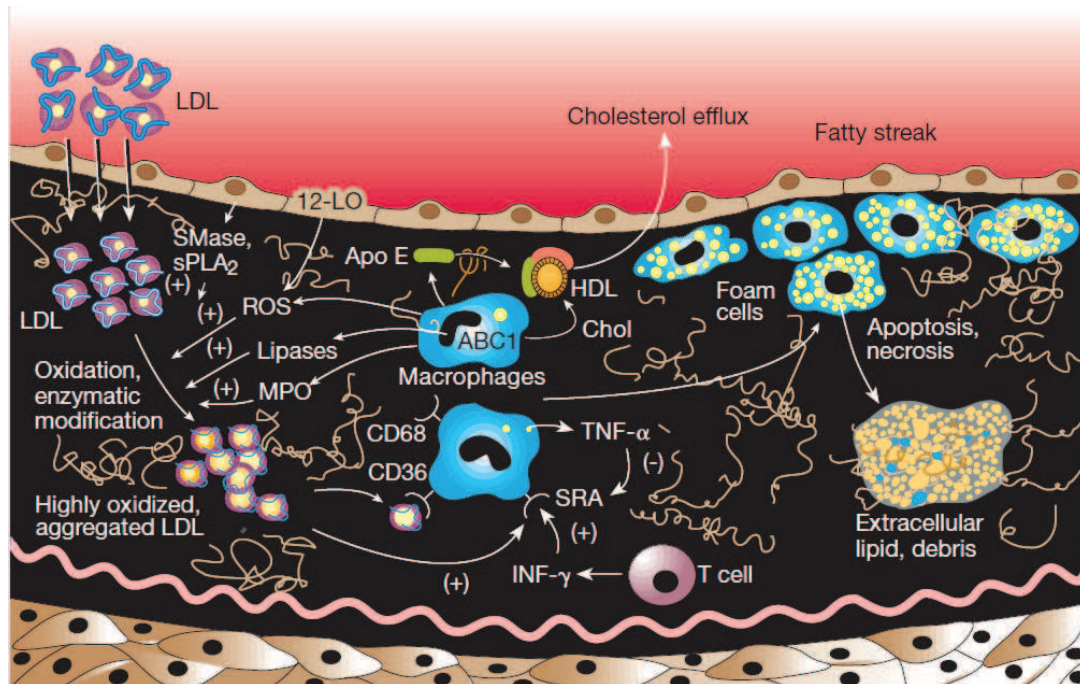


Figure 14: Minimally oxidized LDL stimulates endothelial cells to produce adhesion molecules and growth factors; recruiting monocytes to the vessel wall. LDL can be further oxidized by the generation of ROS by endothelial cells and macrophages (2).

Lesions throughout this development are histologically classified into eight lesion types depending on the composition and severity of the plaque. Type I, or initial lesions, contain enough lipoprotein to induce an increase in macrophages and foam cells. Type II, or fatty streak lesions, consist of fatty streaks and lipid-laden smooth muscle cells, either at highly susceptible sites (type IIa) or moderate, slow developing sites (type IIb). Type III, or intermediate lesions, contain extracellular lipid droplets and particles that alter the coherence

of intimal smooth muscle cells. Type I, II, and III lesions often occur in people under 30 years of age and are always small and clinically silent (7).

Advanced lesions can be subdivided into five main characteristic types: type IV through VIII. Type IV (atheroma) and type V lesions may cause clinical manifestations and become fatal if they reach a size that sufficiently obstructs the vessel. Both type IV and V lesions contain a lipid core, but differ from each other in terms of the fibrous cap. Type IV has a relatively thick cap with a composition similar to a normal intima. The amount of lipid that is segregated at the core determines the degree of lumen narrowing. Due to the outward expansion of the lesion, a type IV lesion will not in most people cause much lumen obstruction. Type V lesions have a fibromuscular cap in which the disrupted tissue has been replaced with accumulated lipid and hematoma or thrombotic deposits. The cap has a greater proportion of rough endoplasmic reticulum-rich smooth muscle cells and collagen fibers. However, in this lesion type, outward expansion is replaced by narrowing of the lumen. Type VI lesions are most often associated with clinical manifestations and fatal outcomes. Histologically, these lesions include one or more surface defect, hematoma, and thrombosis. Lesion type VII (previously type Vb) consists of calcified lesions while lesion type VIII (previously type Vc) consist of fibrous tissues with a visible core (7).



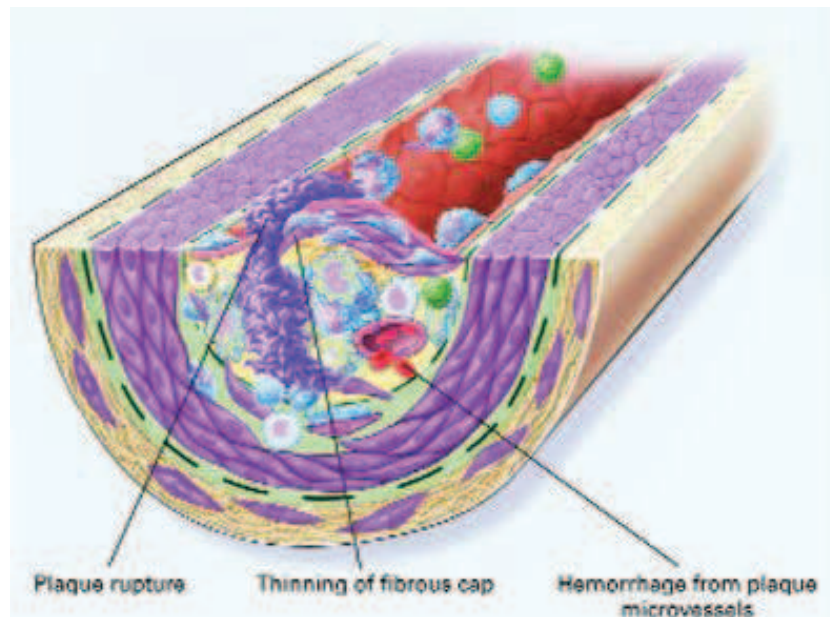


Figure 15: Development of an unstable fibrous plaque. Rupture of the fibrous cap can lead to thrombosis and occurs at thin fibrous caps covering the lesion. Influx and activation of macrophages can lead to the degradation of matrix, leading to hemorrhage. (8)

There are no definite correlations between a lesion's composition and size, or between the degree of lumen obstruction and clinical manifestations. All advanced lesions may obstruct the lumen to the point of producing a clinical event.



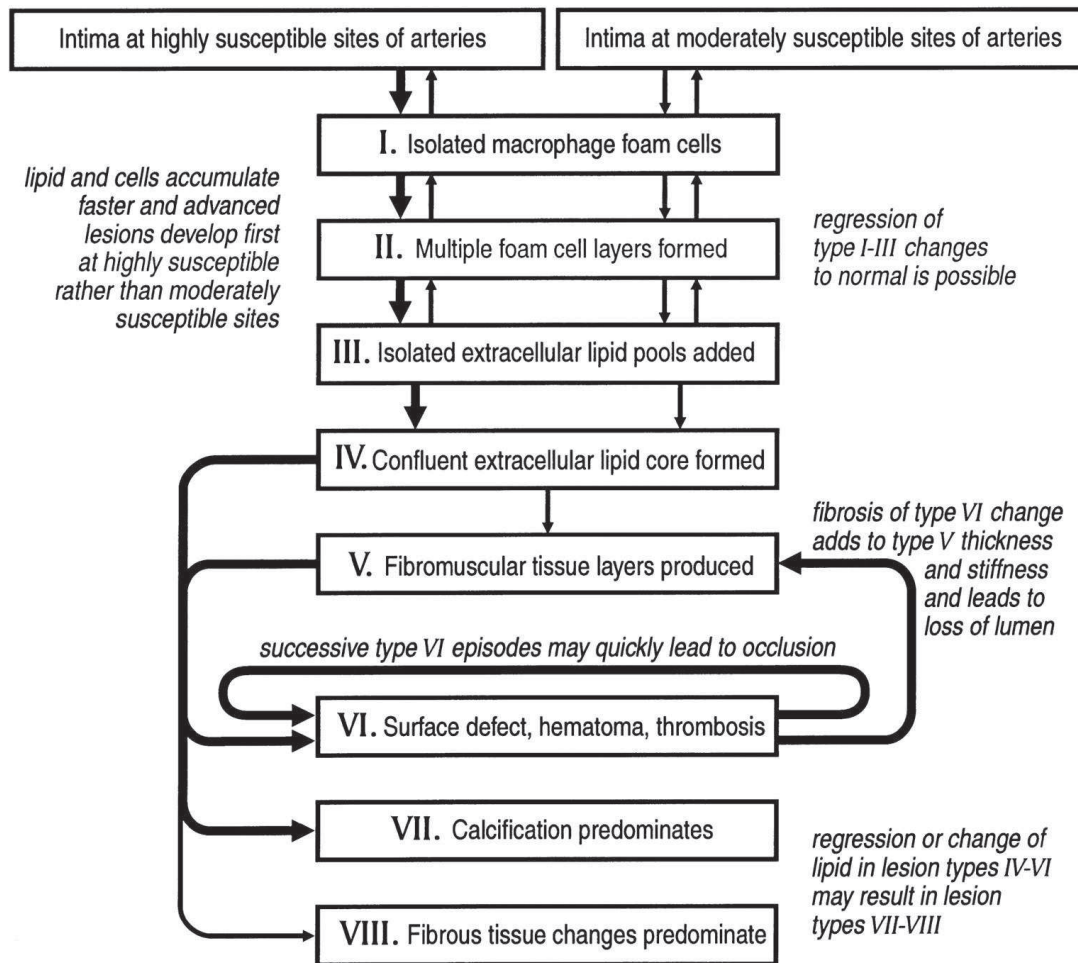


Figure 16: Histological characteristics describing the development of atherosclerotic lesions. Evolution of lesions from type I to type IV and the different pathways to develop advanced lesions (7).

### V.3 Generation of oxidative stress and inflammation in Atherosclerosis.

Atherosclerosis is a condition plagued by an overwhelming systemic increase in oxidative stress and inflammation. However, by consequence atherosclerosis also further generates products of oxidative stress and inflammation. Studies have shown relationships between atherosclerosis and its risk factors, such as hypercholesterolemia, smoking, and inactivity and the development of an overproduction of oxidative stress and inflammation. The major components involved in oxidative stress and inflammation in atherosclerosis include

macrophages, immune cells, lipids, endothelial cell function and activation, adhesion, and smooth muscle cell proliferation and transformation.

The second part of this chapter will detail the ways in which oxidative stress and inflammation play a role in the pathogenesis of atherosclerosis and plaque disruption. We have limited this section to focus on the macrophages, lipids, endothelium, and adhesion molecules, as they are the aspects most relevant to the experimental studies that follow this chapter.

### **V.3a Role of oxidative stress and inflammation in macrophages.**

Macrophages play a key role in both the initiation and progression of atherosclerosis. Macrophages contribute to the oxidative and inflammatory local environment through the production of reactive oxygen species (ROS) and cytokines. There are several different populations of macrophages with diverse functions and specific responses to certain pathologies. The two major macrophage classes involved in atherosclerosis include M1 (classical activation) and M2 (alternative activation) macrophages. M1 macrophages are a result of classical activation in response to IFN $\gamma$  or bacterial moieties such as LPS. This macrophage population is typically considered pro-inflammatory as they produce IL-12, IL-6, IL-1, TNF $\alpha$ , and express cytokine receptors such as IL-1R on the plasma membrane surface. M2 macrophages are alternatively activated in response to IL-4 and IL-13. These cells strongly express scavenger, mannose, and galactose receptors and express anti-inflammatory markers such as Arginase 1 (ARG1), Ym1 and Ym2, Fizz1, and MRC1 (8).

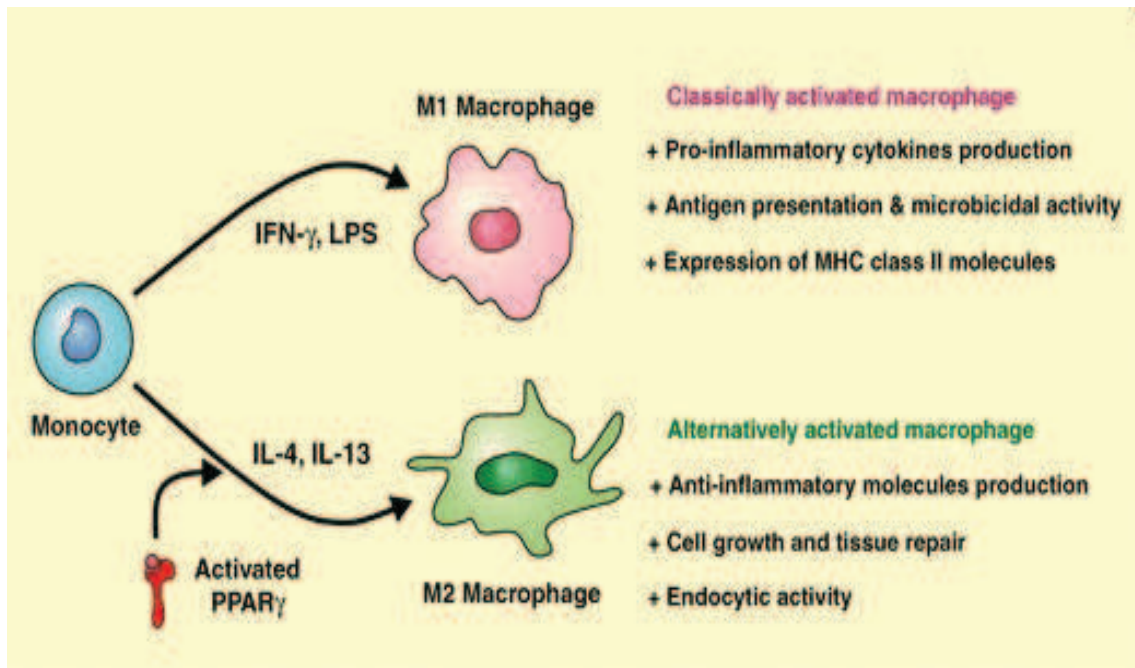


Figure 17: Two pathways of macrophage differentiation. Depending on the cytokine induction, monocytes can differentiate into M1 (pro-inflammatory) or M2 (anti-inflammatory) macrophages (9).

A delicate balance must exist between M1 and M2 macrophages in order to initiate proper healing. In atherosclerosis, there is a chronic imbalance between macrophage phenotypes, as well as cycles of active recruitment and modulation of phagocytic activity. Macrophages can adopt different phenotypes depending on stimulation and can represent a variety of activated phenotypes rather than one stable subpopulation. Some studies have determined the plasticity of macrophages by demonstrating their ability to switch from one functional phenotype to another in response to the local environment (10, 11). The risk factors for atherosclerosis, such as physical inactivity (12), smoking (13), and high blood pressure (14), elicit a highly oxidative and inflammatory response, leading to a more oxidative and inflammatory environment, possibly inducing this change. Young plaques in mice contain a large proportion of M2 phenotype, as demonstrated by an increase in ARG1, possibly due to a reparative,

rather than progressive, action in atherosclerosis. This reparative function is evident *in vitro*, as both M2-conditioned and ARG1-expressing media induced a strong proliferation of VSMC (15). However, as the disease progresses, M2 macrophages are replaced by M1 macrophages as the prevailing macrophage phenotype in lesions (15). The ratio of accumulated M2/M1 in ApoE<sup>-/-</sup> atherosclerotic plaques is inversely proportional to the progression of lesion size (15).

Increased superoxide plays a large role in the initiation and progression of atherosclerosis. ROS derived from NADPH oxidases, such as superoxide, are strongly correlated with the severity of atherosclerosis (16). NADPH oxidase activity in monocytes/macrophages and its resulting superoxide production contribute to atherosclerotic lesion formation. A study by Vendrov et al, showed that the absence of functional NADPH oxidase in bone marrow derived monocytes/macrophages decreased total aortic atherosclerotic lesion area in ApoE<sup>-/-</sup> mice, which was additionally correlated with a decrease superoxide production in the aorta (17). The improvement in lesion area could have occurred due to a reduction in foam cell formation. Both superoxide and monocytes/macrophages can induce LDL oxidation (oxLDL) (18), which results in the unregulated uptake of this lipoprotein by macrophages, leading to foam cell formation.

### **V.3b Role of oxidative stress and inflammation on endothelial dysfunction**

The endothelium is a single layer of cells that line the luminal surface of blood vessels. It functions as a border between circulating blood components and the vessel, and regulates vascular tone via mediators such as nitric oxide (NO), prostacyclin, endothelin-1, and thromboxane A2. Endothelial dysfunction, which results in impaired NO bioavailability leading to reduced vasodilation, is an initial step of atherogenesis and is believed to occur as a result of accumulating free oxygen radicals.

Superoxide, derived from NADPH oxidase, has been implicated in endothelial dysfunction (19). In rats with chronic heart failure, acetylcholine-induced relaxation was reduced along with increased aortic superoxide generation and p47phox protein expression compared to control rats (20). Numerous studies indicate that direct inactivation of NO by  $O_2^{\cdot -}$  represents a key event responsible for impaired NO bioactivity (21, 22). As superoxide is able to decrease the availability of NO, an important anti-atherosclerotic regulator of vascular tone and blood flow, an increase in SOD may improve the outcomes of atherosclerosis. SOD deficient or knockout mice exhibit enhanced  $O_2^{\cdot -}$  and impaired NO bioactivity (23–25). Exogenous SOD improves vascular relaxation response to NO (22) and improves NO-mediated arterial relaxation in atherosclerotic animals (26). A SOD mimetic was able to reverse CHF in dogs by improving coronary blood flow response to acetylcholine (27).

In addition, the reaction of NO and superoxide results in the generation of peroxynitrite ( $ONOO^-$ ): a powerful and highly reactant oxidant. Peroxynitrite may trigger apoptosis in endothelial cells leading to vascular pathophysiology (28, 29). It may also contribute to the development of early lesions, and create reactive nitrogen species that further progress lesions (30). Peroxynitrite is able to regulate signaling pathways in the endothelium, modifying the vascular response to atherogenic stimuli (31). Vendrov (17) et al, showed that NADPH oxidase knockout mice display decreased  $ONOO^-$  production and delayed vascular remodeling in response to an increase in shear stress (17).

Endothelial NO synthase (eNOS) can produce superoxide rather than NO if there is a deficiency of either cofactor tetrahydrobiopterin ( $BH_4$ ) or L-arginine. A lack of  $BH_4$ , which occurs through its oxidation by  $ONOO^-$ , causes an electron flow interruption through the eNOS domain, leading to the generation of superoxide over nitric oxide (32). Treatment of

BH<sub>4</sub> to patients with hypercholesterolemia or hypertension successfully restored endothelial function, emphasizing the role of eNOS uncoupling as a major player in vascular disease (33, 34). The importance of NO in endothelial homeostasis is evident in eNOS knockout mice that develop defective vascular remodeling and enhanced vascular thrombosis (35). It is further demonstrated that eNOS deficiency in atherosclerotic prone mice accelerates lesion formation (36).

Myeloperoxidase (MPO), an enzyme expressed most abundantly in neutrophils, is able to produce hypochlorous acid from hydrogen peroxide during the neutrophil's respiratory burst. In addition, it can consume NO and compete with L-arginine within the eNOS complex. Levels of MPO have been shown to be strong and independent marker of endothelial dysfunction (37).

Xanthine oxidase (XO) produces O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub>. Diseased human vessels from patients with CAD show an increased production of O<sub>2</sub><sup>•-</sup>, resulting from an enhanced expression and activity of XO (38). Vasodilation is improved in patients with hypercholesterolemia (39), CAD (38, 40), and chronic heart failure (41, 42) when XO production is inhibited by the addition of allopurinol or oxypurinol.

### **V.3c Role of oxidative stress and inflammation on adhesion**

An early stage of atherosclerosis involves the recruitment of circulating inflammatory cells into the subendothelial space. This process is mediated by cellular adhesion molecules (CAMs) which are expressed on the vascular endothelium and on circulating leukocytes, especially monocytes, during an inflammatory response (See Figure 18). The abnormal interaction that develops during the progression of atherosclerosis involves platelets, vascular

cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), and E- and P-selectin.

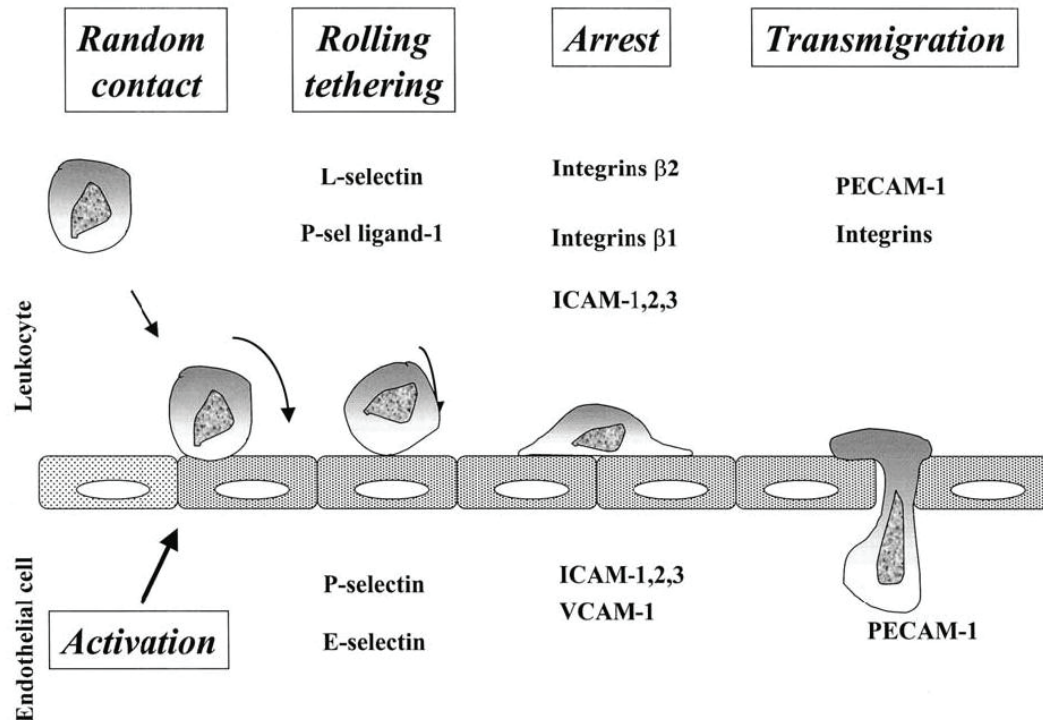


Figure 18: Leukocyte-endothelial cell interaction during the beginning steps during the development of atherosclerosis. Adhesion molecules play a role in this process (43).

Activated platelets may contribute to lesion formation, as they expedite the migration of inflammatory cells to lesion sites and release pro-inflammatory cytokines and chemoattractants (44). Platelet adhesion may be an initiating event, as it has been shown to occur before leukocyte recruitment to arterial wall and precedes activation of NF- $\kappa$ B, VCAM-1 and MCP-1 (45). It has been demonstrated *in vitro* that platelets trigger the secretion of chemokines, express adhesion molecules, and promote adherence of leukocytes in endothelial cells (46, 47). The release of inflammatory mediators forms an inflammatory environment that can lead to cell activation and cell recruitment. *In vivo*, platelet recruitment occurred at lesion-prone sites.



Located on the surface of activated platelets, as well as on activated endothelial cells, is cell adhesion molecule P-selectin. Activated platelets in the circulation are able to initiate an acute inflammatory response by initiating P-selectin expression on endothelium and stimulate leukocyte rolling on vessel wall. P-selectin, in combination with E-selectin (located on endothelial cells) has been shown to increase with the level of severity of atherosclerosis in humans (48). A deficiency of both P- and E- selectin was shown to reduce aortic sinus fatty streaks, delay lesion expansion, and reduce calcification in LDLR deficient mice. (49) Moreover, injections of P-selectin-expressing platelets into ApoE<sup>-/-</sup> mice accelerated the development of lesions (50).

VCAM-1 and ICAM-1 are both cytokine-inducible Immuno-globin gene superfamily members that are expressed by the aortic endothelium in areas predisposed to atherosclerosis (51, 52). VCAM-1 seems to have a predominate role in lesion initiation (53, 54), while more current studies have found that ICAM-1 has a larger role in mature lesion progression (55, 56). In hypercholesterolemic animals (51, 52), both VCAM-1 and ICAM-1 are upregulated before early foam cell lesions, notably at the periphery. However, VCAM-1 expression is limited to lesions and lesion-prone areas, whereas ICAM-1 expression can extend outside the aorta and lesion-prone areas (51). In addition, serum levels of both VCAM-1 and ICAM-1 have been associated with increasing levels of disease severity (48, 57). In young adults with advanced atherosclerotic plaque, intima-media thickness was associated with higher levels of sICAM-1. It was also shown that higher than average sICAM-1 levels corresponded to increased carotid artery stenosis and coronary artery calcification (58).



## V.4 Non-invasive methods for plaque vulnerability investigation

There are several types of imaging modalities to assess complications of cardiovascular disease including ultrasound (including intravascular IVUS for coronary lesions), computed tomography, MRI, PET, and optical (OCT) imaging. This chapter will focus on the use of magnetic resonance imaging as a non-invasive method for assessing plaque vulnerability. The following section will introduce molecular imaging of inflammation and oxidative stress in the context of atherosclerosis.

### V.4a Magnetic Resonance Imaging (MRI)

Magnetic Resonance Imaging (MRI) is an imaging technique that is based upon the sensitivity to the presence and properties of protons mainly from water, which makes up 70-90% of most tissues and fat. As the chemical environment and amount of water protons can vary with disease and injury, MRI can be used as a sensitive diagnostic method. The nucleus of the hydrogen atom is a single positively charged proton, which generates a magnetic field. When placed in a strong external magnetic field, it experiences a turning force. When radio-frequency pulses are applied, the net macroscopic magnetization of protons spins out of alignment. The relaxation to equilibrium is directed by two exponential time constants: T1, the longitudinal relaxation time describing spin-lattice interactions; and T2, the transverse relaxation time describing spin-spin interactions. Within a 1.5 Tesla magnetic field, fluids tend to have long T1s (400-1500ms), and fat-based tissues have short T1s (100-150ms). T2 is always shorter than T1s for a certain tissue. T2s for fluids (700-1200ms) are longer than water-based tissues (40-200ms) and fat-based tissues (10-100ms). Magnetic inhomogeneities affects spin-spin relaxation time causing it to become shorter; this is known as T2\* or susceptibility effects. In T1-weighted images, tissues with long T1 give the weakest signal,

while the opposite is true for T2-weighted images: tissues with long T2 give the highest signal intensities.

There are two main types of pulse sequences: spin echo (SE) and gradient echo (GE). In SE, two RF pulses create the echo in order to measure signal intensity. GE sequences measure signal intensity by using a single RF pulse followed by a gradient pulse to create the echo, thus making them more sensitive to magnetic field inhomogeneities. Multi-echo GE sequence enables the measurement of the T2\* in the tissue, whereas a multi-echo SE sequence is used for T2 measurement. Contrast agents for MRI are able either to decrease proton T1 and T2 relaxation times (i.e. paramagnetic agents, such as gadolinium chelates) or to induce local modification of the proton magnetic environment (i.e. superparamagnetic agents): their effects are generally visualized using T1 and T2\* GE sequences, respectively.

#### **V.4b Imaging in Atherosclerosis.**

Macrophage imaging throughout various stages of atherosclerosis progression in experimental animals and humans could provide novel mechanistic insights into atherosclerosis. The use of MRI can characterize the plaque composition at the cellular and molecular level, and offer an assessment of plaques severity from early stages of subclinical disease to the overall extent of burden in symptomatic patients.

#### ***Inflammation and macrophages***

Plaques with ongoing active inflammation may be characterized by the extensive accumulation of macrophages. A popular method for visualizing inflammation is via the accumulation of macrophages assessed by superparamagnetic iron oxide particles.

Superparamagnetic iron oxide (SPIO)-based particles are composed of an iron oxide core surrounded by a dextran or polymer coat. Particles can range from ultrasmall (USPIO) (20-

50nm diameter) to micrometer-sized (MPIO) (0.9-8 $\mu$ m). Active intraplaque macrophages will phagocytize iron oxide particles that migrate into atherosclerotic plaques. In order for the USPIOs to reach the macrophages, they must diffuse through the abnormal endothelium usually associated with plaque. Therefore, small USPIOs with long circulation times are critical in order to obtain enough intraplaque macrophage uptake (59). USPIO particles have been evaluated experimentally (60, 61) and clinically (62, 63) as markers of activated monocytes/macrophages. Iron oxide particles shorten T2 and T2\* relaxation time, creating hypointense (black) areas [on T2- and T2\*-weighted sequences].

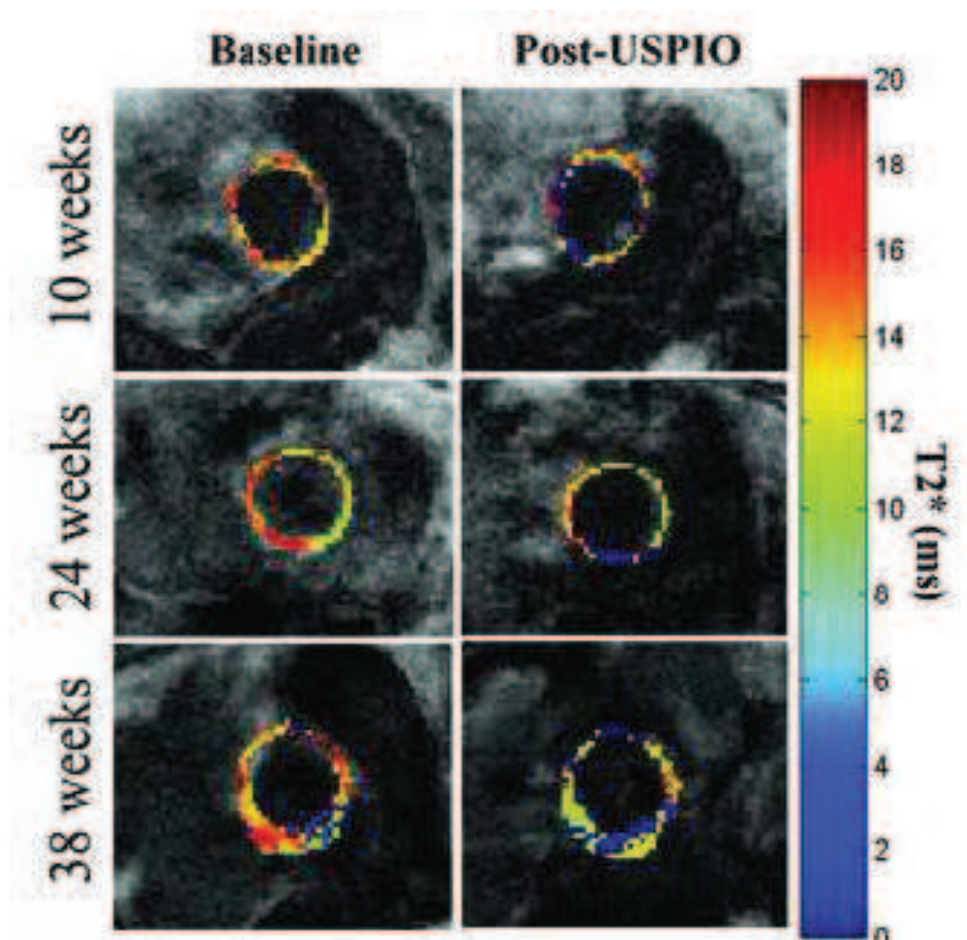


Figure 19: Overlay of T2\* maps of the vessel wall on anatomic images. Post-USPIO images allow detection of macrophage accumulation, which advances with age (64).

In addition to accumulation of macrophages within the plaque, rate of apoptosis is also important to the severity of atherosclerosis. Advanced lesional apoptosis is associated with the development of vulnerable plaques (65), as matrix metalloproteinase (MMPs) are released during cell death. Intraplaque macrophage apoptosis has been linked to vulnerable plaque due to the release of MMPs during cell death. Mouse models of atherosclerosis show that MMP-9 induces plaque progression, cap thinning (66), and secretion of inflammatory mediators. An imaging target developed by the pharmaceutical company Guerbet to target MMP-9 shows that the MR signal increase in the vessel walls of rabbits are associated with histological evidence of MMP activity (67, 68). A labeled MMP inhibitor (MPI) used in ApoE<sup>-/-</sup> mice was able to detect more advanced lesions (69, 70) and maybe a better imaging agent compared to apoptosis-sensing agents (annexin V) (70).

Oxidative stress. Activated macrophage, smooth muscle cells, and endothelial cells increase the production of ROS that can promote cellular damage and serve as a regulator of inflammation. Myeloperoxidase (MPO) has recently become attractive target for molecular imaging as it is present within atherosclerotic plaques and can contribute to atherogenesis by catalyzing oxidative stress in the vascular wall (71). Nahrendorf et al (72) was the first to demonstrate that MPO-Gd (myeloperoxidase sensor bis-5HT-DTPA(Gd)) could noninvasively be used to detect MPO activity in a mouse model of myocardial I/R injury. This study also was able to quantify the anti-inflammatory effects of atorvastatin treatment following injury. It was further shown that MPO-Gd could detect MPO activity within the atherosclerotic plaques of rabbits, confirmed by biochemical assays and histopathological evaluations (73). As MPOs are expressed in active macrophages, the MPO activity may be a more predictive response to plaque vulnerability than the mere presence of macrophages.

MPO imaging may have broad applications in the imaging assessment of cardiovascular disease in humans (74).

MRI can assess oxidative stress through the measurement of hemoglobin. The iron contained within hemoglobin is a potent generator of free radicals that can oxidize LDL (see chapter III.2b). Iron can be released in cases such as intraplaque hemorrhage; an indicator of plaque progression and severity. Extracellular ferric hemoglobin generates T<sub>1</sub> high signal intensities reflected by a pro-oxidant environment (75). In a model of oxidant-producing anoxic-anoxia, an increase in paramagnetic molecules (such as free radicals, iron, or deoxyhemoglobin), was associated with a decrease in T2-weighted images and an increase in T1-weighted images (76).

#### **V.4c Animal models of advanced lesions**

Atherosclerosis is a complex disease that develops over the lifespan. Because of this, there is a need to develop animal models in order to understand the molecular and cellular pathways involved the pathogenesis of atherosclerosis, including diagnostic protocols and therapeutic interventions.

Several mouse models have been developed to observe the full development of atherosclerosis within a reasonable time period under controlled conditions. C57BL/6 mice are susceptible to atherosclerosis and tend to develop small lesions in the aortic root when fed a high fat diet. Prolonged diet leads to lesions with cellular debris and collagen (77). ApoE<sup>-/-</sup> mice are bred on a C57BL/6 background and develop spontaneous lesions even when fed a standard chow diet. Lesions are characterized by foamy macrophages in the proximal aorta by 3 months of age and develop complex lesions by 8-9 months. These complications can be



accelerated by a high-fat/high-cholesterol (HF/HC) diet leading to advanced lesions with a fibrous cap, small necrotic cores, and lipid deposits (78) (See Figure 20). The ApoE<sup>-/-</sup> mouse is often bred with other strains in order to study a specific aspect of the disease. Breeding with LDLR<sup>-/-</sup> (ApoE<sup>-/-</sup>/LDLR<sup>-/-</sup>) mice leads to advanced atherosclerosis with foamy lesions (79). In combination with the eNOS<sup>-/-</sup> (ApoE<sup>-/-</sup>/eNOS<sup>-/-</sup>) mice, atherosclerosis is accelerated, as these mice form aortic aneurysms and ischemic heart disease after 16 weeks on a HF/HC diet (36). Breeding with iNOS<sup>-/-</sup> (ApoE<sup>-/-</sup>/iNOS<sup>-/-</sup>) mice leads to reduced atherosclerosis and lower plasma lipid peroxides (80).

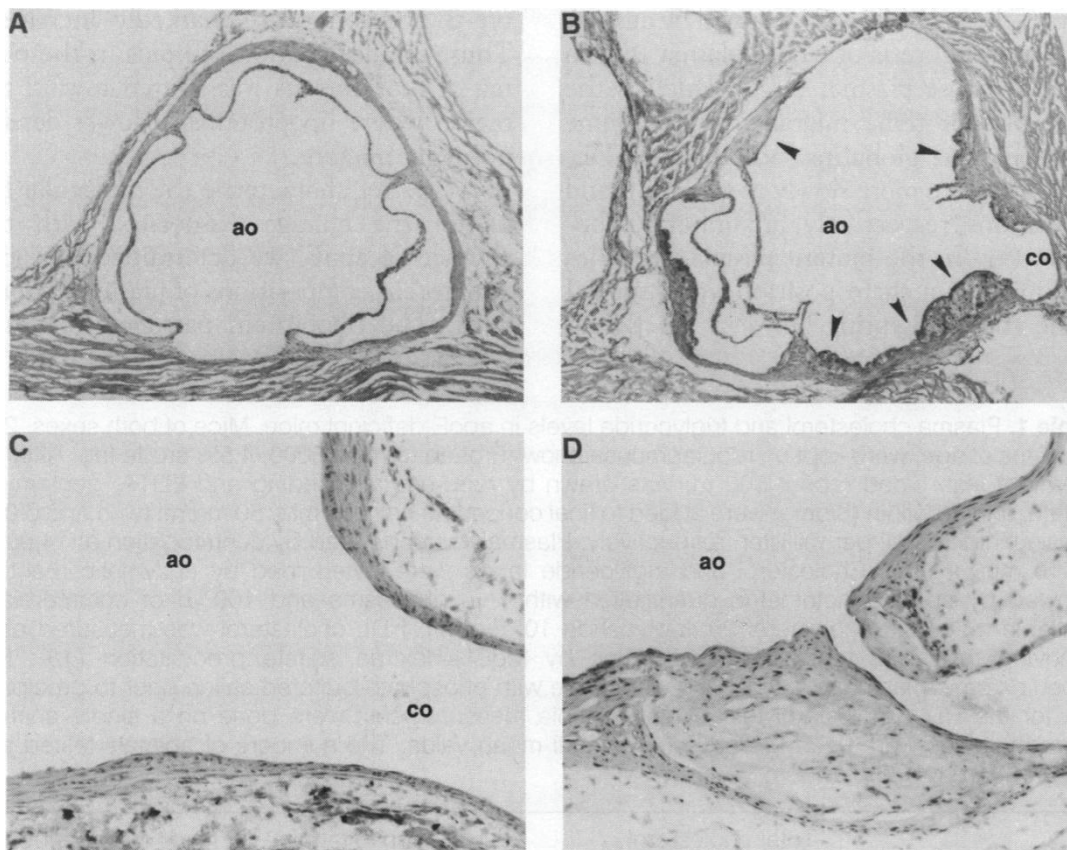


Figure 20: Aortic Sinus of 5 month normal (A) or ApoE<sup>-/-</sup> (B) mouse. Arrows indicate multilayer intimal foam cell deposits. Coronary artery of 5 month normal (C) or ApoE<sup>-/-</sup> (D) mouse. Near total occlusion at ostium of a coronary artery. ao: aorta, co: coronary art (78).

## V.5 EFFECTS OF EXERCISE TRAINING IN ATHEROSCLEROSIS

As chronic exercise training has been shown to have adaptive effects in the body, contrary to the acute, short-lived response of acute exercise, this section will focus on the effects of habitual exercise training on the progression of atherosclerosis.

### V.5a LDL

Circulating LDL is able to permeate the endothelium freely, however at high concentrations LDL tends to accumulate within the intima. This increase in LDL accumulation is an initial event of atherosclerosis. Exercise training is able to reduce plasma LDL concentration, as seen in healthy subjects, hypercholesterolemic men (81), and obese women (82) and children (83). The improvement in LDL concentration may be dependent upon the ability of HDL to effectively clear cholesterol. Exercise training has been shown to increase lipoprotein lipase (84), which accelerates the degradation of triglycerides, resulting in the transfer of cholesterol to HDL. An increase in HDL is evident in many studies comparing trained athletes to sedentary controls (85–87). However, even healthy subjects who underwent detraining showed a decrease in HDL concentrations (87–89).

The oxidation of protein and lipid components of LDL particles is one of the most important initial events in the development of atherosclerosis. LDL can become oxidized by ROS such as myeloperoxidase, 15-LO, and iNOS. Treatment with NADPHox inhibitor or mitochondrial complex I/II inhibitor hindered oxLDL-induced monocyte ROS production (90). Exercise training has been demonstrated to have a role in the reduction of oxLDL, probably through its regulation of ROS. In LDLr deficient mice, exercise training decreased *in situ* superoxide and serum myeloperoxidase, which was associated with a decrease in oxLDL (91). In a human study, exercise training was able to increase LDL resistance to oxidation and decrease already

oxidized LDL (92). In this context, the beneficial effects of exercise may be due to an increase in antioxidant glutathione reductase. GSH has a role in the management of lipid uptake in advanced lesions (93). In fact, LDL resistance was linked to an increase in systemic antioxidant levels of glutathione and glutathione reductase (92). This link between glutathione and oxLDL is supported by an *in vitro* study that found the addition of antioxidants to human monocyte derived macrophages inhibited oxLDL-mediated glutathione loss and GAPDH inactivation, and reduced oxLDL uptake in macrophages (94). In addition, Wang et al (90) found that mild- and moderate-intensity exercise can reduce the diminishing effects of oxLDL on SOD and GSH bioavailability.

#### **V.5b Endothelial dysfunction**

Many studies have demonstrated improved endothelial function as a consequence of exercise training in healthy subjects and those with pre-existing endothelial dysfunction in conditions such as coronary artery disease (CAD), chronic heart failure (CHF), and type 2 diabetes (95, 96). Endothelial dysfunction is characterized by an impaired vasodilation response due to a decrease in NO; both of which can be reversed by exercise training. Exercise training is a mechanical stimulation of the endothelium by shear stress. Shear stress can increase production of NO through eNOS, leading to enhanced vasodilation. Potential mechanisms responsible for the functional adaptations of the endothelium to exercise training include improved prostacyclin and NO bioavailability, and decreased endothelin-1 and angiotensin II.

Clinical and experimental studies have found that exercise training reverses and prevents endothelial dysfunction. In patients with coronary artery disease (CAD), exercise training was shown to correct endothelial dysfunction and improve coronary artery blood flow, by increasing eNOS protein expression (97, 98) and decreasing expression of angiotensin II



type 1 receptor (99). In animal models of atherosclerosis, exercise training was able to increase eNOS expression and improve the relaxation response to acetylcholine (Ach) in obese (100), diabetic (95, 101), and aging (102) mice, hypertensive rats (103), and hypercholesterolemic miniature pigs (104, 105).

In addition to increasing eNOS expression and phosphorylation, exercise training can also prevent NO degradation. As explained previously (see Chapters II.1 and III.2c), NO can be reduced via oxidative stress. Exercise training was able to reduce the activity of the oxidative stress generating enzyme NADPHox (102, 103), IFN- $\gamma$ , and superoxide (101). Improvement of exercise-induced vasodilation is often accompanied by up-regulation of SOD (95, 100, 102). As demonstrated in cultured human aortic endothelial cells, laminar shear stress was able to increase SOD protein content and enzyme activity in a time- and dose-dependent manner (106). The upregulation of SOD through shear-stress could be responsible for the reducing the inhibitory action of O<sub>2</sub><sup>-</sup> on NO (106).

### **V.5c Macrophage/leukocyte recruitment**

As macrophages are able to change their functional phenotype in response to changes in the environment, it is likely that the improved systemic response to oxidative stress and inflammation due to exercise training would have an effect on macrophage phenotypes. Yakeu et al showed that low-intensity exercise upregulated markers of M2 macrophages while downregulating M1 markers (107). This is in agreement with Smith et al who found that the atherogenic M1 cytokines, IFN- $\gamma$ , TNF $\alpha$ , and IL-1 $\beta$  were decreased by moderate-intensity exercise training, while the atheroprotective M2 cytokines, TGF- $\beta$ , and IL-10 were increased (108). Both adipose tissue macrophages and peritoneal tissue macrophages contribute to the immune disruption which is involved in atherosclerosis. Exercise training

was able to change the phenotypic expression of adipose tissue macrophages from M1 to M2 in obese mice on a high-fat diet (11). In addition, exercise training was able to provide protection against an inflammatory response by altering macrophage response (109).

In addition to the change in macrophage phenotype, the amount of macrophage accumulation within the plaque can affect the severity of atherosclerotic lesions. Exercise decreased macrophage and T-lymphocyte infiltration in adventitia, preserved vascular function in aging C57 mice (110). Exercise was able to reduce leukocyte infiltration into the peritoneal cavity. In addition exercise was able to prevent MAPK-induced regulation of proinflammatory mediators by increasing MAPK phosphatase-1 (MKP-1). This is supported *in vitro* as cultured peritoneal macrophages from exercise trained mice showed increased MKP-1 expression, reduced p38 MAPK activity, and reduced secretion M1 cytokines, IL-6, TNF $\alpha$ , and MCP-1 (111).

MMP-9 activity can induce the rupture of advanced atherosclerotic lesions in apoE<sup>-/-</sup> mice (112). ApoE<sup>-/-</sup> mice crossed with MMP-9<sup>-/-</sup> mice exhibit less severe and more stable aortic lesions, accompanied by impaired macrophage infiltration (113). Exercise training was able to decrease MMP activity even in mice on high-fat diet with preexisting atheromata (114). Exercise training was able to suppress the number of macrophages, which occurred with a reduction in MMP-9 areas in plaque. One mechanism to explain the exercise-induced improvement of MMP-9 is via an improvement in oxidative stress: ROS can induce MMP-9 while NO can inhibit it (115–117). This is supported by Shon et al (114) that found a decrease in lipid peroxidation occurred concurrently with a decrease of MMP-9.

### V.5d Adhesion

A healthy endothelium can limit the abnormal adherence of blood cells to the endothelium. When endothelial cells become activated during inflammatory conditions, they express adhesion molecules such as VAM-1 and ICAM-1. These molecules not only assist in the attachment of blood cells, but also signal the recruitment of other blood cells. In response to exercise training, cell adhesion molecules have a reduced ability to adhere in more physically fit and/or healthy individuals. This was demonstrated *in vitro* as peripheral blood mononuclear cell to HUVEC adhesion was reduced in more physically fit subjects than the less-fit subjects in response to a 20-min moderate exercise (118). Meta-analysis of the effects of exercise interventions in patients with CAD found VCAM-1 levels to be significantly lower postintervention (119). In animal studies with hypercholesterolemic rabbits, it was found that exercise training reduced the protein expression of adhesion molecules and iNOS, leading to an improvement in vasorelaxation (120).

As mentioned in the previous section, platelets play an important role in the pathogenesis and progression of CVD. Platelet adhesiveness and aggregability were decreased after exercise training protocol (121–123), and these results were reversed during deconditioning (121). As high levels of LDL and VLDL increase platelet aggregability and secretion (124), it is likely that the improvements in lipoprotein concentrations that occur with exercise training could be responsible for this decrease in platelet activity.

Other possible mechanisms for the improvement in adhesion due to exercise training could be due to increased shear stress, decreased oxidative stress, or improved nitric oxide. Shear stress decreases the expression of VAM-1 and E-selectin. Oxidative stress is closely involved in the regulation of CAM expression. Superoxide produced from NADPH oxidase has been

implicated in increased expression of CAMs (125). Other ROS such as  $H_2O_2$  produces transcription factors such as NF- $\kappa$ B and activator protein-1 (AP-1), which enhance expression of adhesion molecules, such as VCAM-1, ICAM-1, and E-selectin (126). Treatment with antioxidants pyrrolidine dithiocarbamate (PDTC) and N-acetylcysteine (NAC) reduced TNF $\alpha$ -induced VCAM-1, suggesting that the improvement of antioxidants associated with exercise could play the same role (127). In addition, the increase in NO as a response to increased shear stress regulates adhesion markers. As discussed previously (See Chapters II.1 and III.2c) NO regulates the expression of VCAM-1, ICAM-1, and platelets. A study by de Meirelles (123) et al showed that platelet aggregation reduction was associated in an L-arginine-induced improvement in NO.

#### V.5e Plaque instability

The progression of generalized atherosclerosis can be measured by the intima-media thickness (IMT) of the plaque. It is closely related to risk of ischemic events such as myocardial infarction (MI) and stroke (128, 129). Although physical inactivity can increase carotid IMT (130), aerobic exercise capacity has been shown in many studies to be inversely associated with the progression of IMT (131–135). The Los Angeles Atherosclerosis Study found that the most ideal effects were seen in the vigorous exercise group, yet a graded inverse relationship was observed between physical activity and IMT progression, independent of other cardiovascular risk factors (136). Although other studies disagree (137), it has been shown that VO<sub>2</sub>max was the strongest predictor of maximal and mean carotid IMT, plaque height, and surface roughness (138).

In addition to quantifying the IMT, the instability of a plaque determines future consequences, such as unstable angina, acute myocardial infarction, and stroke. As explained previously (see

Chapter V.2), a clinically vulnerable plaque is defined by a thin fibrous cap, a reduction in SMC content, a large lipid core, and an accumulation of inflammatory cells within the lesion. In a study by Fukao et al (*139*), exercise training was able to decrease lesions in the aortic sinus and thoracoabdominal aorta in ApoE<sup>-/-</sup> mice on high-fat diet. The author observed a strong negative correlation between atherosclerotic areas and the mean running distance (*139*). Many other animal models have described the beneficial effects of exercise training on plaque stability by showing improved characteristics such as increased thickness of fibrous cap, decreased macrophage accumulation, increased collagen accumulation, increased SMC content, and decreased fatty streak formation (*117, 140–144*). Often times these changes occur without altering risk factors such as high blood pressure, high cholesterol, or dietary changes (*140, 141, 143*).

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## Chapter VI

**Article #2: MRI evidence of exercise-induced improvement of oxidative stress and inflammation in the brain of old apoe<sup>-/-</sup> mice.**

## Chapter VI.

### **MRI evidence of exercise-induced improvement of oxidative stress and inflammation in the brain of old high fat fed ApoE<sup>-/-</sup> mice.**

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## ABSTRACT

**Background:** Stroke shares pathological hallmarks with atherosclerosis, such as increased inflammation and oxidative stress. Physical activity reduces these risk factors, but its effect on stroke is less documented. Using in-vivo magnetic resonance imaging (MRI) and biological markers of oxidative stress and inflammation, we investigated a model of progressive brain damage in old ApoE<sup>-/-</sup> mice fed a high fat diet, and then sought to determine the beneficial effects of exercise on oxidative stress and inflammation in brain as a treatment option in this aging atherosclerosis mouse model.

**Methods and Results:** Using MRI, we demonstrated that aging and high fat diet induce a high occurrence of vascular abnormalities in the brain of ApoE<sup>-/-</sup> mice over 70 weeks old which were associated with increased oxidative stress and inflammation. Exercise training was able to significantly reduce both MRI-detected abnormalities (present in 71% of untrained *vs.* 14% of exercise trained mice) and markers of oxidative stress (MDA: -43%, AOPP: -15%) and inflammation (TNF $\alpha$ : -17% and IL-1 $\beta$ : -20%) in the brain. In parallel, exercise decreased insulin resistance and aortic, heart, and systemic oxidative stress and inflammation, but significant associations were only seen within brain markers.

**Conclusions:** We demonstrated the occurrence of vascular brain damage in an aging model of atherosclerosis and showed that exercise training is able to reduce this outcome. In parallel, exercise decreased oxidative stress and inflammation directly in the brain. Non-invasive imaging appears to be an essential tool to evaluate neurovascular damage in the aging brain and the protective effect of exercise.

Key Words: imaging, exercise, inflammation, oxidative stress, atherosclerosis

## INTRODUCTION

The pathology of stroke develops in a similar manner to that of atherosclerosis, as increased inflammation and oxidative stress are evident in both conditions. In the context of aging and disturbed lipid trafficking, the brain is highly susceptible to ROS-induced damage, due to its high rate of oxidative metabolism and relatively low levels of antioxidant enzymes<sup>1</sup>. It is possible that blood brain barrier permeability<sup>2</sup>, as well as the macrophage infiltration that is common in atherosclerosis, could also affect the brain vessel function in the aging process<sup>3,4</sup>. Therefore, risk factors for atherosclerosis could be involved in the development of inflammatory conditions in the brain, and ultimately lead to stroke<sup>5</sup>.

Risk factors for cardio- and cerebro-vascular diseases (CVD) are associated with an increase in inflammatory cytokines, oxidative stress, and accumulation of macrophages that can lead to pathophysiological changes in arteries resulting in vascular dysfunction, further increasing the risk of disease<sup>6-8</sup>. Exercise training has been well documented in the amelioration of oxidative stress and inflammation in CVD<sup>9</sup>. Exercise training is able to reduce oxidative stress by increasing antioxidant capabilities and by maintaining nitric oxide metabolism<sup>10</sup>. An increase in antioxidants has also been shown to occur in conjunction with a decrease in lipid peroxidation and protein oxidation<sup>11</sup>. Exercise training could also be an impetus for an anti-inflammatory environment as aerobic exercise has been associated with lower circulating levels of pro-inflammatory markers<sup>12</sup>. Exercise is also connected with improvement in general metabolic conditions such as lipid profile and insulin resistance. As shown by Pellegrin et al<sup>13,14</sup>, the beneficial effects of exercise training on atherosclerosis have been established in ApoE<sup>-/-</sup> mice.

In old ApoE<sup>-/-</sup> under high fat/high cholesterol (HF/HC) diet, acute events such as paralysis, hemiplegia, and sudden death have been observed (unpublished observation) which are highly suggestive of cerebrovascular diseases. We used aortic and brain MRI to further

characterize these clinical observations and to relay information concerning the systemic and local effect of exercise in this advanced vascular and metabolic model. We hypothesized that exercise training could ameliorate vascular-induced abnormalities in the brain and decrease inflammation: two conditions that suggest high risk of stroke or other neurovascular complications. In-vivo exploration of inflammation using iron oxide nanoparticles enable the detection of focal macrophage accumulation<sup>15</sup>. Exercise training could concomitantly prevent the systemic complications of atherosclerosis, even in the context of several high-risk conditions, i.e. aging and lipid/cholesterol overload. Therefore we also evaluated systemic and brain markers in old and control young-adult ApoE<sup>-/-</sup> mice.

## METHODS

*Ethical approval.* All animal procedures conformed to the European regulation for animal use and this study was approved by the local ethics committee of the institution.

*Animals:* ApoE<sup>-/-</sup> mice (C57BL/6 background, Charles-River, France) were fed a HF/HC diet (Western Diet, 21% fat, 0.5% cholesterol, SAFE, Augy, France) starting at 8 weeks of age. All animals were maintained on a 12 hour light-dark cycle and were supplied with food and water *ad libitum*. After careful maintenance of health conditions during one year (Guerbet, Animal Care Unit), 60±1 weeks old mice were randomly divided into 2 activity groups. Mice in the exercise trained group (O-ExT) were individually housed in cages equipped with a 12.5 cm metal running wheel (HAGEN-61700, Montreal, Canada) and digital magnetic counter (model BC906 Sigma Sport, Neustadt, Germany), while the untrained (O-UT) group had a standard cage. Young-adult ApoE<sup>-/-</sup> mice (Y-CON; age = 10 weeks) under the same high fat diet (starting at 8 weeks of age) served as controls. During the 12 weeks of training, the distance run and the general health (i.e. tumors, skin irritations) of the mice were noted three times a week. The exclusion criterion was overall bad health of the animal (i.e. tumors, skin irritations).

*Cholesterol and metabolic measurements:* Total blood cholesterol was assessed using Amplex Red Cholesterol Assay Kit as instructed by Invitrogen (Carlsbad, CA). One week before MRI, intraperitoneal insulin tolerance test (IPITT) was performed on 6h-fasted mice. Mice were injected intraperitoneally with 0.75mU/g body weight of insulin. Blood was taken by tail puncture immediately before and at different time points after injection for measurement of plasma glucose.

To determine metabolic muscle adaptations to physical activity, skeletal muscle citrate synthase activity was determined using soleus muscle homogenate by the method of Shepherd and Garland<sup>16</sup>.

*MRI Protocol:* Mice were randomly selected to undergo the imaging protocol. The mice were anesthetized by isoflurane (2% for the induction and 1% to maintain anesthesia) (TEM SEGA, Lormont, France). Cardiac and respiratory rates were monitored throughout the session and body temperature was maintained using a circulating heated water blanket at  $37\pm 1^{\circ}\text{C}$ . MRI acquisitions were performed on a 4.7T Bruker magnet (Ettingen, Germany).

For ascending aorta imaging, double cardiac and respiratory gated acquisitions were obtained as previously described<sup>17</sup> (see Supplemental Data) with a homemade gating system developed in Matlab (The MathWorks Inc, Natick, MA). ECG signals were collected via the three electrodes placed on the paws and respiratory signals were collected via a pressure sensor placed on the abdomen.

For brain imaging, a birdcage coil of 72-mm inner diameter was used for RF transmission and a surface coil anatomically shaped to the mouse head for reception (Rapid Biomedical, Wurzburg, Germany).

Brain T2-weighted spin-echo images were acquired using a RARE sequence on both axial and coronal planes. (See Supplemental Data Figure #1 for imaging protocol). To further characterize the neurovascular lesions, an extended brain MRI protocol was performed in a separate set of old untrained ApoE<sup>-/-</sup> mice under HF/HC diet (n=10). Hemorrhage and stroke were assessed respectively by baseline T2 and T2\* imaging. T2\* quantification was obtained using a multi-slice multi-echo gradient echo (MGE) sequence (See supplemental data for more detail). Gadolinium chelate (Gd-DOTA, 1 mmol/kg, Guerbet, Aulnay-sous-Bois,

France) was then injected to observe possible blood brain barrier (BBB) leakage, followed by P904 injection and the 48 hour post-contrast T2/T2\* imaging.

The total duration of the MRI protocol was less than 2 hours. The protocol was then repeated 48 hours after the injection of iron nanoparticles (P904, 1 mmol Fe/kg, Guerbet, Aulnay-sous-bois, France) for vessel wall inflammation assessment, followed by the brain imaging acquisition (supplemental Figure S1).

*MRI Analysis.* For brain analysis, areas of interest on pre-contrast T2/T2\* images and post-gadolinium images were first visually categorized based on the size of abnormal areas and the number of slices affected. We scored changes in pre- and post- contrast (48 hours post-P904) on T2/T2\* images and gadolinium leakage on T1-weighted images. Briefly, a score of 1-4 was given for abnormalities seen on pre-contrast images and a score of 1-4 was given for changes seen on post-contrast images (See Table 1 for scoring assessment).

For analysis of the aortic arch, inner and outer vessel wall contours were delineated and vessel wall area calculated. T2\* mapping of pre- and post-contrast series was performed using Matlab (The MathWorks Inc, Natick, MA) on interleaved MSME images. The vessel wall regions of interest (ROIs) were used for the analysis of aortic T2\* on both pre- and post-contrast images as described by Sigovan et al<sup>18</sup>.

*Dissections:* Following the second imaging session, mice were anesthetized by an intraperitoneal injection of pentobarbital (50mg/kg, Dolethal®, Vétquinol, Lure, France) and blood was collected by cardiac puncture. The heart was transcardially perfused for 70 sec with 9% NaCl. The brain, heart, ascending-descending aorta, and soleus were removed. Sections to be used for biological assays were stored at -80°C until assessment.

*Immunohistochemistry:* Brain samples were harvested and fixed in a paraformaldehyde solution during 1 hour followed by sucrose for 24 hours and preserved at -80°C until processing. Four successive 15µm thick sections for 3 MRI locations were assessed with standard Hematoxylin Eosin, F4/80 immunostaining for macrophages, and IgG immunostaining for blood brain barrier permeability, as previously described<sup>19</sup>.

*Biological analysis:* All tissues were kept frozen and homogenized with a 10% v/w buffer (PBS 1x + 0.5mM EDTA). Homogenates were centrifuged at 4°C for 4 min at 1500g for protein content and MDA analysis, and again at 4°C for 10 min at 12000g for the remaining analyses. Supernatants were frozen at -80°C. Protein concentrations were determined spectrophotometrically (Biophotometre, Eppendorf, Germany) using a BCA kit according to instructions (Sigma, St Louis, USA).

*Oxidative stress and Inflammation assessment:*

Oxidative stress markers AOPP and MDA were measured in plasma, heart, and brain. Nitrotyrosine was assessed in plasma. Antioxidant markers catalase, GPX, and SOD were measured in plasma, heart, and brain. FRAP and NOx were measured in plasma. All reagents used for biochemical assays were purchased from Sigma Aldrich. A detailed description of oxidative stress markers can be found in supplementary data.

*Inflammatory Markers.* TNFα (BD Biosciences, San Jose, CA) and IL-1β (RayBiotech, Inc, Norcross, GA) were assessed in plasma and brain supernatant using a commercially available mouse enzyme-linked immunosorbent assay kit, according to manufacturer instructions. NF-κB/p65 activity (IMGENEX, San Diego, CA) was assessed in plasma according to manufacturer instructions.

*Statistics:* Analyses were conducted using Statistica (version 8.0, Statsoft, Tulsa, OK, USA). Results are presented as mean ± SEM. For each parameter, a minimum of 7 mice per group



was used. Statistical comparisons between 3 groups (O-UT, O-ExT, Y-CON) were performed by one-way analysis of variance followed by Bonferroni post hoc test. Pearson's coefficient correlations were used to determine the associations between plasma markers, brain markers, and distance run. Logrank test was used for survival curve analysis. Statistical significance was determined by a P value of less than 0.05.

## RESULTS

### *Animal characteristics and general effects of exercise*

107 old mice and 20 young-adult mice (Y-CON) were originally included in the protocol. Of the 55 mice that died, 32 were in the old untrained group (O-UT) and 10 were in the old trained group (O-ExT) (Figure 1A). Seven of them (13% of all deaths) presented with acute mono- or hemi-plegia, a possible symptom of stroke.

An additional 6 mice died during insulin resistance test and MRI, and 3 mice were excluded at dissection because of large tumors.

After the induction and training period, 43 old mice (19 O-UT:  $72.4 \pm 2.4$  weeks and 24 O-ExT:  $71.8 \pm 1.9$  weeks) and 20 young-adult mice (age:  $20 \pm 0$  weeks) were used for biological assessment (Table 2). The O-ExT mice ran  $17.8 \pm 15.3$  km/week. The training effect was supported by higher CS activity in the soleus of O-ExT mice compared to O-UT mice (see Table 2). The training effect was also evident in metabolic parameters such as insulin sensitivity and plasma cholesterol levels in the O-ExT versus O-UT mice (see Table 2). Despite higher plasma cholesterol levels in O-ExT, young ApoE<sup>-/-</sup> mice had higher insulin sensitivity than old untrained ones, as observed during IPITT (Table 2). Exercise training was able to decrease the mortality rate of the old mice. The O-ExT mice had a significant higher survival rate compared to the O-UT mice (77 vs. 49%,  $p=0.03$ ; Figure 1A).

*Presence of multiple neurovascular lesions in old sedentary ApoE<sup>-/-</sup> mice under HF/HC diet*

As observed by *invivo* MRI and histology, there were significant abnormalities in the brain vasculature of old sedentary ApoE<sup>-/-</sup> mice under HF/HC diet (Figure 2). On pre-contrast images, several dark areas on T2 images and T2\* images indicated hemorrhage in a large number of mice. Post-gadolinium images indicated the presence of periventricular blood brain barrier leakage and endothelial permeability in old ApoE<sup>-/-</sup> mice (Gadolinium score of  $2.7 \pm 0.25$ ). Comparing the pre-contrast to post-contrast P904 images (Figures 1 and 2), it was evident that there was also an accumulation of iron oxide nanoparticles suggesting an accumulation of macrophages and phagocytic activity in the same area. These signs of neuro-inflammation evident on both post-contrast T2\* and T2 images were confirmed on histology (Figure 2). Disorganized brain parenchyma was seen in the middle ventral zone. Hemorrhage occurred in the periphery of mid-cerebral artery and in some cases could spread to the brain parenchyma (see Figure 2 F-H). This abnormal area on both T2/T2\* images and post-contrast gadolinium images corresponded to positive staining for IgG and F4/80, which would indicate BBB leakage (endothelial permeability), and macrophage accumulation in the choroid plexus and fornix fimbria (supplementary Figure S2). Visually, there was also some evidence of vesicular aggregates which could indicate foam cell development.

*Brain MRI in trained versus untrained ApoE<sup>-/-</sup> mice.*

As observed by brain MRI in the old mice (O-UT, n=7; O-ExT, n=7), there were significant abnormalities in the brain vasculature of O-UT mice (71% of mice) compared to O-ExT mice (14% of mice). Comparing the pre-contrast images (respective scores,  $1.71 \pm 0.19$  vs.  $1.29 \pm 0.19$ ;  $p=0.12$ ) to post-contrast images of the sedentary mice, it was evident that there was an accumulation of iron oxide nanoparticles suggesting phagocytic activity and

inflammation (See figure 1) with a significantly higher post-contrast score in O-UT mice compared to O-ExT mice ( $2.92 \pm 0.49$  vs.  $1.69 \pm 0.44$ ;  $p < 0.05$ ).

*Exercise-induced changes in brain and systemic oxidative stress and inflammation* (Tables 3 and 4, and Figure 3). In brain, MDA and AOPP were decreased in response to exercise training ( $p < 0.01$  and  $0.1$ , respectively). Moreover, there was an increase in brain catalase ( $p = 0.035$ ), and a decrease in IL-1 $\beta$  and TNF $\alpha$  in O-ExT compared to O-UT mice ( $p < 0.05$ ). Brain concentrations of oxidative stress marker MDA were significantly higher in old mice compared to young-adult mice, as well as pro-inflammatory markers, TNF $\alpha$  and IL-1 $\beta$  ( $p < 0.01$ ). Brain antioxidant markers, catalase, GPX, and SOD, were significantly lower in old mice than in young-adult mice.

Plasma MDA and AOPP decreased in response to exercise training in the old mice (respectively  $p < 0.05$  and  $p < 0.1$ ), whereas antioxidant markers NOx and SOD increased (respectively  $p < 0.05$  and  $p < 0.1$ ) (see Figure 3 and Table 4). Pro-inflammatory markers, TNF $\alpha$  and IL-1 $\beta$  were also decreased in response to exercise training in old mice ( $p < 0.05$ ). Plasma AOPP and MDA were different in old untrained mice compared to young-adult mice ( $p < 0.01$ ). Pro-inflammatory markers, TNF $\alpha$  and IL-1 $\beta$  were also significantly different in old mice compared to young-adult mice ( $p < 0.001$ ).

*MRI and biological vessel wall response.* Old mice had a larger vessel wall area than the young-adult mice ( $3.37 \pm 0.3$  vs.  $2.87 \pm 0.03$  mm<sup>2</sup>;  $p < 0.01$ ). In the old trained mice, vessel wall area was reduced compared to untrained mice (see Figure 4). Concerning T2\* measurements, pre-contrast values in old mice were lower than in young mice, confirming more complex plaque composition<sup>20</sup>. The exercise trained group had an increase in pre-contrast T2\* measurement compared to O-UT mice, suggesting a less complex plaque composition (see

Figure 4). Post-contrast T2\* was lower than pre-contrast values, indicating the presence of iron particles and phagocytic activity in the vessel wall. However, there was no significant difference between the changes in pre-contrast to post-contrast in any group. In the aorta, old mice had more TNF $\alpha$ , IL-1 $\beta$ , SOD, and AOPP than young-adult mice. O-UT mice, had more TNF $\alpha$  and AOPP in the aorta than their corresponding ExT counterpart (see Figure 4), but less IL-1 $\beta$  ( $p < 0.05$ ) and SOD ( $p < 0.1$ )

*Heart markers.* Heart concentrations of AOPP were higher in old untrained mice compared to young-adult mice. O-UT mice had lower GPX and SOD than young-adult mice. O-ExT mice had higher levels of SOD and lower AOPP than O-UT mice (see Table 4).

#### *Correlations.*

Some of the inflammation and oxidative stress markers were correlated within the brain (see Table 6). However, none of the brain markers were correlated with corresponding plasma markers (see supplementary data, Table 1S).

## **DISCUSSION**

Stroke shares similar pathological features with atherosclerosis, such as increased inflammation and oxidative stress<sup>REF</sup>. In this study, we assessed the effects of exercise on brain vascular abnormalities associated with atherosclerosis in ApoE<sup>-/-</sup> mice using *in vivo* MRI and biological markers. We found that ApoE<sup>-/-</sup> mice exhibit an age-related increase in brain abnormalities and endothelial permeability, confirmed by both MRI and histology, which were associated with an increase of both inflammation and oxidative stress markers in the brain. In addition, age-associated changes were evident in atherosclerotic traits such as increased metabolic disorders, aortic plaque instability, oxidative stress, and inflammation. We found that exercise training, possibly via its oxidative stress and inflammation lowering capabilities, reduced brain macrophage infiltration, limited inflammation and oxidative stress

in the brain, and improved metabolic conditions, thereby improving health status and life expectancy. The results from this study also indicate that brain MRI could be beneficial in the evaluation of atherosclerosis burden and the associated vulnerability.

The ApoE genotype has protective properties against atherosclerosis, including its involvement in lipoprotein metabolism, antioxidant properties, and immunosuppressive properties<sup>21</sup>. For this reason, the knockout of this gene in this transgenic mouse model accelerates atherosclerotic plaque development via an increase in plasma cholesterol and inflammation<sup>22</sup>. As shown by Bonomini et al<sup>23</sup>, ApoE<sup>-/-</sup> mice have increased oxidative stress and inflammation compared to control mice, which further increases with age. All these phenotypic changes are likely responsible for the very low survival (49% over 12 weeks) of our ApoE sedentary mice compared with C57BL6 at the same age (i.e. 60-70 weeks)<sup>24</sup>.

The brain is more susceptible to oxidative damage than other tissues due to its high content of poly-unsaturated fatty acids, high rate of oxidative metabolism, and low levels of antioxidants<sup>1</sup>. Moreover, in agreement with the studies of others<sup>25,26</sup>, we found that aging further increases oxidative stress in the brain. A possible explanation could be that aging is associated with an overproduction of oxidative stress without a concomitant production of antioxidants. For example, Chan et al<sup>27</sup> showed that aging was associated with an impairment of oxidative phosphorylation and decreased activity of several electron transport chain complexes<sup>27</sup>, leading to overproduction of ROS. Further supporting our study, increased lipid peroxidation and decreased SOD have been found to be associated with increasing ageREF! Oxidative stress in the cerebral vasculature plays a critical role in the pathogenesis of ischemic brain injury (such as a compromised BBB and macrophage accumulation)REF!!.

BBB disruption, as measured in this study by the extent of gadolinium leakage in T1-weighted MRI, is a sign of endothelial permeability<sup>28</sup>. During conditions of inflammation, macrophages are able to cross the BBB and infiltrate the CNS parenchyma<sup>29</sup>. In our study,

the location of gadolinium leakage corresponded with the accumulation of macrophage detected *in vivo* after injection of iron nanoparticles by T2-weighted MRI and post mortem by histology. Brain parenchymal macrophages (microglial cells) are able to react to an inflammatory stimulus such as hemorrhage<sup>30</sup>. Hemorrhage within the cerebrovasculature, which was detected on some pre-contrast T2\* images of old mice, could possibly explain the observed symptoms of hemi- and monoplegia.

### *Exercise Training.*

Our results show that even in an atherosclerosis-prone mouse model of aging, exercise training was able to reduce risk factors for atherosclerosis and stroke, including macrophage accumulation, oxidative stress, inflammation, and metabolic parameters. Voluntary wheel running (VWR) was the chosen method of exercise training in this study. As mice are naturally active, VWR provides a stress-free way to exercise, as opposed to forced treadmill training and swimming which may induce a stress response<sup>31</sup>. In these studies, stress may be a confounding factor that could lead to an increase in oxidative stress and inflammation. In several studies, this VWR model of training has been shown to sufficiently produce cardiovascular adaptations such as heart and left ventricular hypertrophy, and an increase in muscle oxidative capacity<sup>32</sup>. Although the distance run by the mice in this study was low compared to healthy C57 mice<sup>32</sup>, it was sufficient to increase muscle citrate synthase activity, a commonly used marker of an adaptation to habitual exercise<sup>33</sup>.

In our study, the old sedentary mice had a higher rate of mortality compared to the exercise trained mice. This is consistent with previous reports that physical inactivity is an independent predictor of mortality in animals<sup>34</sup> and humans<sup>35</sup>. This suggests that exercise training was responsible for the augmented survival rate of old ApoE<sup>-/-</sup> mice. This is further supported by a study that found that voluntary exercise was sufficient to extend survival and decrease neuronal damage after a short episode of forebrain ischemia<sup>36</sup>.

Physical fitness was found to be associated with preserved brain health<sup>37,38</sup>. In treadmill trained rats, induced brain injury was less severe as a consequence of decreased cerebral permeability and improved brain integrity<sup>39</sup>, which may be due to an increase in collagen IV expression and improved BBB function<sup>40</sup>. In a study on aging women, physical fitness was associated with higher cerebrovascular conductance and cognitive function<sup>41</sup>. In this population, cerebrovascular function was negatively correlated with oxidative stress and positively with nitric oxide<sup>42</sup>. It is possible that this mechanism could also explain why the mice that were exercise trained had less brain abnormalities.

The importance of antioxidants in the prevention of neurodegenerative disease is becoming evident. In our study, old exercise trained mice had higher levels of antioxidants such as SOD and catalase. In aging rats with mitochondrial degeneration and DNA and RNA damage in the brain, supplementation of antioxidants lipoic acid and acetyl-L-carnitine prevented the structural decay of hippocampal mitochondria<sup>43</sup>. In addition, the role of inflammation in neurodegeneration is evident as the supplementation of anti-inflammatory agents has shown a protective role. Individuals that consume diets high in omega-3 fatty acids or take anti-inflammatory agents such as NSAIDs have a decreased risk of neurodegenerative diseases<sup>44</sup>. Although we did not test specific anti-inflammatory cytokines, exercise training reduced pro-inflammatory markers which could be due to a concomitant increase in anti-inflammatory cascades<sup>35,45</sup>. In agreement with our study, exercise was also shown to reduce brain IL-1 $\beta$  in a mouse model of Alzheimer Disease<sup>46</sup> and brain inflammation in response to stroke<sup>47</sup>, possibly through an increase in anti-inflammatory pathways.

Moreover, improvement of brain health has been shown to be related to systemic improvement of cardiovascular health, lipid-cholesterol balance, inflammation<sup>45</sup>. In our study, we found that exercise training was able to improve the overall effects of atherosclerosis. MRI revealed a decrease in vessel wall size in the mice that ran compared to

sedentary mice. This is in agreement with others that found a decrease in lesion size<sup>13,48,49</sup>. In a study by Pellegrin et al<sup>13</sup>, swim-trained ApoE<sup>-/-</sup> mice showed a decrease in macrophage accumulation, along with an increase in smooth muscle cell content suggesting a more stable plaque. Although there was no change in macrophage accumulation in our study, we found that exercise trained mice had a more stable plaque, as gauged by the higher value of MRI T2\* values. As demonstrated by Sigovan et al<sup>18</sup>, T2\* measurements strongly correlate with histological assessment of complex lesions including large lipid deposits, calcification, and macrophage accumulation.

Lastly, this aged atherosclerotic model showed different vascular mechanisms at work in the brain and in the “system/periphery”. Our study differs from Drake et al<sup>50</sup>, who concluded that systemic inflammation in young animals could “prime” the brain prior to stroke presentation. However, in the present study, few correlations were found in oxidative stress and inflammatory markers between the brain and aorta, heart, or plasma. This lack of correlation between brain and plasma suggests that systemic inflammation may not be entirely clinically relevant to identify brain injuries in susceptible patients.

In conclusion, we found that risk factors for stroke and atherosclerosis, which involve chronic systemic oxidative stress and inflammation, are associated with brain vascular lesions in old ApoE<sup>-/-</sup> mice. All together our results demonstrate that 12 weeks of moderate physical activity was able to improve survival in aged ApoE<sup>-/-</sup> mice. In addition, this voluntary wheel running protocol decreased brain disorders (BBB leakage and macrophage accumulation) and aortic plaque size, and increased aortic plaque stabilization. The decrease in oxidative stress and inflammation directly at the brain level as a result of exercise training could be responsible for the reduced prevalence of lesions. Finally, on the basis of this study, non-invasive imaging such as MRI appears to be an essential tool i) to evaluate neurovascular risk



in the brain of atherosclerotic patients and ii) to measure therapeutic intervention such as physical exercise.

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## FIGURES LEGENDS

**Figure 1:** (A): Survival Rate ( $p=0.03$ ); (B): T2 brain MRI in trained versus sedentary old mice showing a hypointense region (arrow) demonstrating inflammation in the left periventricular fornix fimbria, i. Trained pre-injection, ii. Trained 48h-post-injection, iii. Sedentary pre-injection, iv. Sedentary post-injection ; (C): Pre and Post-Contrast Scores for old mice. \* Significantly different from old untrained mice (O-UT); † Significantly different from Pre-Contrast  $P<0.05$ . ‡  $P=0.12$  vs. O-UT group.

**Figure 2:** Brain MR images of a sedentary ApoE<sup>-/-</sup> mouse. Pre (A) and Post (B) native T2 image showing hyposignal and heterogenous regions around the choroid plexus representative of vascular sequelae (arrows); Pre (C) and Post (D) T2\* maps, with an increase of the hyposignal region on post-contrast, suggesting inflammation (arrows); Pre (E) and Post (F) Gadolinium, the enhancing bright zone showing BBB leakage in the same area; Positive F4/80 staining confirming macrophages in this area (G); Positive IgG staining (H) in the same locations confirming MRI findings of BBB leakage.

**Figure 3:** Oxidative Stress (lipid oxidation, MDA) and Inflammation (TNF $\alpha$  and IL-1 $\beta$ ) markers in the plasma (A-C) and in the brain (D-F). \* Significantly different from young control mice (Y-CON); † Significantly different from old untrained mice (O-UT);  $P<0.05$ .

**Figure 4:** MRI Pre- and Post- Contrast T2\* measurements (A). MRI T2\* measurements (B). Aortic arch vessel wall area (C). TNF $\alpha$  (D), IL-1 $\beta$  (E), and protein oxidation (AOPP) (F) in

aorta. \* Significantly different from old untrained (O-UT) mice; † Significantly different from young control mice (Y-CON); ‡ Significantly different from Pre-Contrast.  $P < 0.05$

## TABLES

**Table 1:** Visual lesion characteristics

| Score | Pre-contrast Image                                                                  | Post-contrast Image                                                                        |
|-------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------|
| 1     | No abnormality                                                                      | No observable change from pre-contrast                                                     |
| 2     | Small lesions (pixel $> 10$ ) on $\leq 2$ slices                                    | Change in SNR and increase in dark region size from pre-contrast on $\leq 2$ slices        |
| 3     | $> 2$ slices or medium size lesion ( $10 < \text{pixels} < 20$ ) on $\leq 2$ slices | Medium change in SNR and increase in dark region size from pre-contrast on $\leq 2$ slices |
| 4     | Large lesion ( $> 20$ pixels) on $> 2$ slices                                       | Major change in SNR and increase in dark region size from pre-contrast on $> 2$ slices     |

SNR: Signal to noise ratio

**Table 2:** Effect of age and exercise training on body weight, citrate synthase activity, cholesterol and insulin resistance

|                          | YOUNG-<br>ADULT |            | OLD              |  |
|--------------------------|-----------------|------------|------------------|--|
|                          | Control         | Untrained  | Exercise Trained |  |
| Body weight (g)          | 32.7±1.5        | 38.5±1.5*  | 40.2±1.5*        |  |
| Citrate Synthase         |                 |            |                  |  |
| (nmol/min/ mg protein)   | 3.68±0.2        | 6.76±0.8*  | 8.50±0.2*†       |  |
| Cholesterol              |                 |            |                  |  |
| (mmol/L)                 | 5668±515        | 5027±121   | 4581±168*†       |  |
| Insulin Resistance       |                 |            |                  |  |
| (% change from baseline) | -20.7±8.1       | -11.6±2.2* | -22.3±2.8†       |  |

Insulin resistance was estimated as the % change of glycemia (vs. baseline) during insulin tolerance test. Means±SEM. \* Young significantly different from Old; † Significantly different from Untrained; P<0.05.

**Table 3:** Effect of age and exercise training on brain markers of oxidative stress

|                                                                   | YOUNG-<br>ADULT  | OLD             |                     |
|-------------------------------------------------------------------|------------------|-----------------|---------------------|
|                                                                   | Control          | Untrained       | Exercise<br>Trained |
| AOPP-brain<br>( $\mu\text{mol}/\text{mg}$ protein)                | 14.5 $\pm$ 1.1   | 15.0 $\pm$ 1.4  | 12.7 $\pm$ 1.2‡     |
| Catalase-brain<br>( $\mu\text{mol}/\text{min}/\text{mg}$ protein) | 174.1 $\pm$ 14.6 | 65.5 $\pm$ 6.3* | 90.2 $\pm$ 11.5*†   |
| GPX-brain<br>( $\mu\text{mol}/\text{min}/\text{mg}$ protein)      | 16.8 $\pm$ 1.14  | 7.43 $\pm$ 1.5* | 7.47 $\pm$ 1.2*     |
| FRAP-brain<br>( $\mu\text{mol}/\text{mg}$ protein)                | 62.2 $\pm$ 4.1   | 56.9 $\pm$ 5.2  | 50.7 $\pm$ 4.3      |
| SOD-brain<br>( $\mu\text{mol}/\text{min}/\text{mg}$ protein)      | 9.56 $\pm$ 0.8   | 6.96 $\pm$ 1.1* | 7.16 $\pm$ 0.9*     |

AOPP: protein oxidation; MDA: lipid peroxidation; GPX, catalase and SOD: antioxidant enzymes activities; FRAP: ferric reducing antioxidant power.\* Significantly different from Young control; † Significantly different from Old untrained; P<0.05. ‡ P<0.1 vs. O-UT.

**Table 4:** Effect of age and exercise training on aorta and heart markers of oxidative stress and antioxidants

|                                                                   | YOUNG-<br>ADULT  | OLD                  |                  |
|-------------------------------------------------------------------|------------------|----------------------|------------------|
|                                                                   |                  | Exercise             |                  |
|                                                                   | Control          | Untrained            | Trained          |
| SOD-aorta<br>( $\mu\text{mol}/\text{min}/\text{mg}$ protein)      | 9.27 $\pm$ 2.1   | 26.2 $\pm$ 1.7*      | 19.4 $\pm$ 2.0*‡ |
| AOPP-heart<br>( $\mu\text{mol}/\text{mg}$ protein)                | 28.5 $\pm$ 2.4   | 75.3 $\pm$ 1.8*      | 68.4 $\pm$ 1.6*† |
| Catalase-heart<br>( $\mu\text{mol}/\text{min}/\text{mg}$ protein) | 831 $\pm$ 76     | 2492 $\pm$ 59*       | 2033 $\pm$ 54*†  |
| GPX-heart<br>( $\mu\text{mol}/\text{min}/\text{mg}$ protein)      | 451.2 $\pm$ 11.5 | 322.7 $\pm$ 7.8<br>* | 338.4 $\pm$ 7.0* |
| SOD-heart<br>( $\mu\text{mol}/\text{min}/\text{mg}$ protein)      | 32.7 $\pm$ 0.9   | 24.1 $\pm$ 0.7*      | 32.2 $\pm$ 0.6†  |

AOPP: protein oxidation; MDA: lipid peroxidation; GPX, catalase and SOD: antioxidant enzymes activities; FRAP: ferric reducing antioxidant power.\* Young significantly different from Old; † Significantly different from Untrained;  $P < 0.05$ . ‡  $P < 0.1$  vs. O-UT.

**Table 5:** Effect of age and exercise training on plasma markers of oxidative stress, antioxidants and inflammation

|                                      | YOUNG-<br>ADULT  |                   | OLD                 |
|--------------------------------------|------------------|-------------------|---------------------|
|                                      | Control          | Untrained         | Exercise<br>Trained |
| AOPP<br>( $\mu\text{mol/L}$ )        | 202.6 $\pm$ 9.1  | 152.4 $\pm$ 11.8* | 131.0 $\pm$ 10.7*‡  |
| GPX<br>( $\mu\text{mol/L/min}$ )     | 123.0 $\pm$ 17.7 | 113.4 $\pm$ 14.5  | 102.4 $\pm$ 13.7    |
| Nitrotyrosine<br>( $\text{nmol/L}$ ) | 25.6 $\pm$ 3.8   | 50.6 $\pm$ 11.0*  | 66.4 $\pm$ 8.9*     |
| NFkB<br>( $\text{pg/mL}$ )           | 97.4 $\pm$ 13.6  | 102.3 $\pm$ 15.6  | 84.1 $\pm$ 12.2     |
| NOx<br>( $\mu\text{mol/L}$ )         | 22.3 $\pm$ 1.3   | 22.6 $\pm$ 1.0    | 27.3 $\pm$ 1.1*†    |
| SOD<br>( $\mu\text{mol/mL/min}$ )    | 37.1 $\pm$ 1.0   | 32.3 $\pm$ 1.4*   | 36.7 $\pm$ 1.2‡     |

AOPP: protein oxidation; Nitrotyrosine: protein nitration; NFkB: inflammation markers; GPX and SOD: antioxidant enzymes activities; NOx: nitric oxide metabolism (nitrites + nitrates); FRAP: ferric reducing antioxidant power.\* \* Young significantly different from Old; † Significantly different from Untrained; P<0.05. ‡ P<0.1 vs. O-UT.

**Table 6:** Correlations between brain markers of oxidative stress and inflammation

| Variables           | -with               | N  | Pearson's   |         |
|---------------------|---------------------|----|-------------|---------|
|                     |                     |    | Correlation | p-value |
| MDA-brain           | IL-1 $\beta$ -brain | 26 | .4294       | 0.029   |
| Catalase-brain      | FRAP-brain          | 27 | .4526       | 0.018   |
|                     | SOD-brain           | 27 | .5734       | 0.002   |
|                     | GPX-brain           | 26 | .5045       | 0.009   |
| TNF $\alpha$ -brain | IL-1 $\beta$ brain  | 27 | .5076       | 0.007   |
| FRAP-brain          | SOD-brain           | 27 | .5515       | 0.003   |
|                     | GPX-brain           | 26 | .5151       | 0.007   |
| SOD-brain           | GPX-brain           | 26 | .6831       | 0.000   |



## SUPPLEMENTAL MATERIAL

### Supplemental Methods

#### *Aortic MRI.*

The ascending and descending aorta were identified using reference axial slices. A bright-blood cine-mode FLASH sequence was used to locate the aortic arch. The reference slices were acquired with a gradient echo (GE) sequence with the following parameters: TR/TE = 1R-R interval/6.7 ms; field of view = 3.8 x 3.8 cm; matrix = 256 x 256; bandwidth = 25 kHz; slice thickness = 1.1 mm; number of averages = 1, number of slices = 1. The oblique slice was placed perpendicular to the ascending aorta directly above the sinus to avoid flow artifacts.

A black-blood multi-gradient echo sequence was used to image USPIO accumulation using the same slice number and positioning, spatial resolution, partial echo acquisition, and the following parameters: minimum repetition time, 742 milliseconds (achieved by setting the gating system between 3 and 5 RR intervals depending on the animal's heart rate); 4 echoes; band width, 79.3 kHz; and number of averages, 2. The sequence was performed twice with two different TE: 3.1 milliseconds, followed by 4.0 milliseconds. The acquired 8 echo images were interleaved to allow a better sampling of the T2\* decay curve.

T2 RARE and T2\* MGE sequence positioned using standard MRI brain anatomical references for careful pre and post-contrast registration were acquired. The RARE sequence was used with the following parameters: TR/TE = /ms; field of view = 2 x 2 cm; matrix = 256 x 256; bandwidth = 85 kHz; slice thickness = 1 mm; number of averages = 1, number of slices = . The MGE sequence was used with the following parameters: TR/TE = /ms; field of view = 2x2 cm; matrix = 256 x 192; bandwidth = kHz; slice thickness = 1mm; xxx echoes and echo interval = ms, number of averages = 1, number of slices = . For BBB permeability

assessment, a T1-weighted MGE sequence with identical geometrical parameters was acquired before and 10 minutes after Gd-DOTA injection with the following parameters: TR/TE= /ms; field of view = 2 x 2 cm; matrix = 256 x 192; bandwidth = kHz; slice thickness = 1 mm; number of averages = 1, number of slices = .

### **Markers of oxidative stress.**

Citrate Synthase. Briefly, citrate synthesis rate from acetyl coenzyme A and oxaloacetate was determined according to a coupling reaction between coenzyme A and DTNB (5,5'-Dithiobis(2-nitrobenzoic acid). This coupling reaction was spectrophotometrically measured at 412nm for 45 seconds.

Advanced Oxidation Protein Products (AOPP). AOPP were determined in plasma, heart, and brain supernatant using the semi-automated method developed by Witko-Sarsat et al<sup>1</sup>, as previously described<sup>2</sup>. Briefly, AOPP were measured by spectrometry on a microplate reader (TECAN Infinite M200, Mannedorf, Switzerland) and were calibrated with a chloramine-T solution that absorbs at 340 nm in the presence of potassium iodide. The absorbance of the reaction was immediately read at 340 nm on the microplate reader against a blank containing 200 µl of PBS. AOPP activity was expressed as micromoles per liter of chloramines-T equivalents.

Catalase. Catalase activity in the plasma, heart, and brain were determined by the method of Johansson and Borg<sup>3</sup>, using hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) as a substrate, and formaldehyde as standard. Catalase activity was determined by the formation rate of formaldehyde induced by the reaction of methanol and H<sub>2</sub>O<sub>2</sub> using catalase as enzyme.

Ferric Reducing Antioxidant Power (FRAP). Plasma FRAP was measured by spectrophotometry using the method of Benzie and Strain<sup>4</sup>. Briefly, FRAP concentration was calculated using an aqueous solution of a known  $\text{Fe}^{2+}$  concentration ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ) as the standard. Plasma was mixed with a FRAP working solution at 37°C, containing buffer acetate, 2,4,6-Tris(2-pyridyl)-s-triazine, and ferric chloride ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ).

Glutathione Peroxidase (GPX). GPX in the plasma, heart, and brain was determined by the modified method of Paglia and Valentine<sup>5</sup>, using hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) as a substrate. GPX was determined by the rate of oxidation of NADPH to  $\text{NADP}^+$  after addition of glutathione reductase (GR), reduced glutathione (GSH), and NADPH.

Malondialdehyde (MDA). Although MDA assay shows methodological limitations<sup>6</sup>, it is the most common lipid peroxidation marker and it is still a widely used marker of oxidative stress. Concentrations of plasma, heart, and brain MDA were determined as thiobarbituric reactive substances by a modified method of Ohkawa et al<sup>7</sup>, as previously described<sup>8</sup>.

Nitrite and Nitrate (NOx). The end-products of endothelium nitric oxide, nitrites and nitrates, were measured in the plasma, based on methods previously described<sup>9</sup>. The sum of nitrite and nitrate in the plasma (NOx) is considered an index of nitric oxide production<sup>10</sup>.

Nitrotyrosine. Concentrations of plasma nitrotyrosine, as end product of protein nitration by  $\text{ONOO}^-$ , were measured as previously described<sup>11</sup>.

Superoxide dismutase (SOD). The quantitative determination of the SOD activity was performed on the plasma and brain using the Beauchamps and Fridovich's method<sup>12</sup>, slightly modified by Oberley and Spitz<sup>13</sup>. SOD activity was determined by the degree of inhibition of

the reaction between superoxide radicals, produced by a hypoxanthine-xanthine oxidase system, and nitroblue tetrazolium.

### Supplemental Figures legends

Figure 21S: MR imaging protocol. A pre-contrast imaging (T-0h) protocol was performed on the aorta followed immediately by basic brain imaging. A contrast agent, P904, was then injected. 48 hours later (T-48h), an identical post-contrast aorta imaging protocol was performed for inflammation assessment. This was immediately followed by brain inflammation imaging.

Figure S22: Macrophage accumulation as assessed by F4/80 (A-C), BBB leakage as assessed by IgG (D-F) in central region (top panels), right region (middle panels), and left region (bottom panels). Areas of interest indicated with arrows. On the right, the reference MRI T2 image.

**Supplemental table****Table 1S:** Lack of correlations between brain and plasma markers of oxidative stress and inflammation

| Variables            | -with               | N  | Pearson's<br>Correlation | p-value |
|----------------------|---------------------|----|--------------------------|---------|
| TNF $\alpha$ -plasma | TNF $\alpha$ -brain | 21 | -.1818                   | 0.430   |
| IL-1 $\beta$ -plasma | IL-1 $\beta$ -brain | 22 | .1700                    | 0.449   |
| AOPP-plasma          | AOPP-brain          | 42 | .1482                    | 0.349   |
| MDA-plasma           | MDA-brain           | 27 | -.0811                   | 0.688   |
| SOD-plasma           | SOD-brain           | 27 | .1836                    | 0.359   |

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# Chapter VII

## Conclusions & Perspectives



## **VII. Conclusions and Perspectives**

### **VII.1 Sickle Cell Disease and Trait**

#### **VII.1a Conclusion**

In the first study, *Exercise training blunts oxidative stress in sickle cell trait carriers*, the purpose was to determine the impact of regular exercise training on plasma markers of oxidative stress in SCT carriers. Supporting our hypothesis, we found that there is relatively little difference between SCT carriers and healthy subjects at rest. However, in response to a maximal exercise test, untrained SCT carriers were at higher risk for increased oxidative stress, while the trained counterparts were protected from the increase in oxidative stress. Exercise training is able to improve antioxidant and NO availability which can modulate ROS production. The combination of these factors could decrease endothelial activation, thereby reducing vascular adhesion.

Our study was able to show that SCT carriers have an impaired oxidative response to an acute bout. Although SCT is usually considered to be benign, we demonstrate that under certain conditions, SCT carriers can exhibit similar response to those with sickle cell disease. Although clinical differences exist between SCT and SCD, both are overwhelmed by an increase in oxidative stress. Therefore, the beneficial response to exercise training that was evident in SCT carriers could be translated to SCD patients as well.

#### **VII.1b Perspectives**

As discussed previously, very few studies have been done on SCD patients and the effects of exercise training. The first step in designing a future study should be to determine which patients can be included. The study by Tinti et al (1) used a steady state patient that had not

reported recent vaso-occlusive crises. Using steady state patients would reduce the risk of complications due to exercise training. This assessment would have to be determined by each patient's physician.

Because of the severity of the disease, it is critical to determine an intensity in which symptoms are limited. A low-intensity, gradual buildup of an exercise training program is advised in order to acclimate the patients to the effects of exercise. An exercise protocol as suggested for CVD rehabilitation (2) could be applicable. A 20-minute bout of moderate-intensity exercise did not cause obvious hemorheological changes (3), however it has been suggested that exercise over 20-minutes should be avoided and breaks should occur at signs of fatigue (4). Exercise training, even at low-intensities, is sufficient to improve oxidative stress and inflammation. Consequently, it is probable that low-intensity exercise training in sickle cell disease patients should be used to decrease oxidative stress and inflammation, leading to a reduction in adhesion and vaso-occlusion.

## VI.2 Atherosclerosis

### VII.2a Conclusion

In the second half of this thesis, we discussed the role of exercise training on oxidative stress and inflammation on the progression of atherosclerosis and its risk factors. Using a mouse model of atherosclerosis, we were able to assess the severity of the disease not only in the cardio-vasculature, but within the cerebro-vasculature as well. It is well supported that the lack of apoE, which accelerates the progression of atherosclerosis, is associated with the presence of neurovascular lesions and neurodegeneration. A deficiency of apoE in mice in combination with a high-fat diet not only increases cholesterol levels and plaque severity, but also increases susceptibility to neuropathology, as indicated by the development of

xanthomas in the choroid plexus, an increase in IgGs in the brain, and a disturbance in the BBB.

We found that exercise training alone is able to improve mortality rate in old ApoE<sup>-/-</sup> mice. In combination with this, we showed that exercise training had a beneficial response in the reduction of neurovascular abnormalities. The prevalence of hemorrhage in the sedentary old mice was related to the increase macrophage accumulation in the brain. The cause could be related to an imbalance of oxidative stress and inflammation seen at the systemic and cerebral levels. The addition of exercise training was also able to reduce risk factors to atherosclerosis, as seen in the reduction of aortic vessel area, improved insulin sensitivity, and reduced oxidative stress and inflammation in the plasma and metabolic organs. The combination of these factors could explain the improvement in survival in the old exercise trained atherosclerotic mice. Inflammatory biomarkers are often used to assess the severity of atherosclerosis and neurodegenerative diseases. As our study indicated, the inflammatory activity within the plasma does not completely draw a parallel with the events in the brain. This indicates that plasma markers may not be sufficient to determine the severity of atherosclerosis consequences such as stroke. Because of this, a combination of biomarkers and the use of MRI could more fully estimate the severity of the disease and determine the likelihood of stroke in high risk atherosclerosis patients.

## **VII. 2b Perspectives**

As atherosclerosis is the leading cause of mortality and stroke the third, methods to reduce and monitor these occurrences are vital. The use of non-invasive methods to assess the severity of atherosclerosis and stroke is also critical. Despite advances in our understanding of the pathophysiology and pathogenesis of these diseases, there is still a lack of non-invasive

imaging tools. MRI is the most promising as it can characterize plaque composition and detect thrombus. The development of novel molecular enhancers could target specific cells and molecules specific to the development and progression of plaque. USPIOs are commonly used in experimental models, but are still in the early phases of clinical trials. Being able to adapt our aortic and brain MRI protocol to the clinical level could allow the detection of stroke risk in atherosclerotic patients.

As described in the second half of my thesis, exercise training has beneficial effects on several players of atherosclerosis even in advanced/unstable atherosclerosis. According to the American Heart Association, cardiac rehabilitation programs play an important and efficient role in preventative care for patients with history of myocardial infarction, chronic heart failure, and other forms of CVD. However, cardiac rehabilitation programs remain underused: only 10-20% of the >2 million patients per year participate (5). As supervised, physician-directed exercise training in high risk patients is relatively safe (only 2 fatalities reported per 1.5 million patient-hours of exercise), exercise training should be used as a preventative measure for the development, as well as the progression, of cardiovascular disease.

Further mechanistic explanation is required to understand how exercise training exerts specific beneficial effects to the development of atherosclerosis. Macrophages are dominant players in the progression of atherosclerosis. The balance between pro-inflammatory macrophages (M1) and anti-inflammatory macrophages (M2) are disturbed, leading to the promotion of plaque. As previously explained, oxidative stress and inflammation are regulators of the recruitment and polarization of macrophages. Therefore, an important next step could be to determine the direct effects of exercise training on the macrophage phenotype. We began a pilot study looking at the changes in bone marrow derived

macrophages (BMDM). We began by stimulating BMDM from C57/Bl6 mice with IFN- $\gamma$  (induction of M1) or IL-4 (induction of M2) to verify the proper pro-inflammatory or anti-inflammatory cytokine response. The second step was to add an oxidative or anti-oxidant mimetic to determine whether this could influence the cytokine response (See Annex IX.2a). The promising results from this initial study, lead us to repeat the experiment using the BMDM from exercise trained or sedentary ApoE<sup>-/-</sup> mice. However, because of a technique problem we were unable to properly culture these cells. This study should be reexamined as it would bring a new mechanistic light as to how exercise training can reduce plaque development.

### VII.3 Final Conclusion.

The work of this thesis supported the beneficial effects of exercise training on oxidative stress in two distinct types of vascular disease. These effects were seen at the systemic levels in SCT carriers and ApoE<sup>-/-</sup> mice, as well as in the aorta, heart, and brain of old ApoE<sup>-/-</sup> mice. Regardless of the disease, the effects of exercise training were sufficient to reverse the complications of the disease and render the unhealthy subjects at a closer level to the control subjects.

A possible linking mechanism for the reduction in oxidative stress in both conditions could be related to the role of oxidative stress produced by RBCs. The reduction of oxidative stress evident in SCT could be influenced by HbS. In SCD, HbS produces two times as much oxidative stress as normal Hb, leading to increased adhesion and vaso-occlusion. It is possible that a RBC-induced increase of oxidative stress is at play in atherosclerosis as well. In addition to increased adhesion which can lead to a further accumulation of macrophages within the plaque, hemorrhage can also be involved. Carotid intraplaque hemorrhage in an

atherosclerotic lesion is considered the culprit responsible for clinical symptoms. RBCs accumulate within the plaque after rupture and its phagocytosis can lead to macrophage activation and foam cell formation. RBCs can also increase oxidative stress and inflammation by releasing oxidized lipids and attracting more macrophages into the plaque. This was evident in our study as signs of RBC degradation were evident in the brains of old *Apoe*<sup>-/-</sup> mice (hemorrhage, intravascular thrombus in microvessels). And so, RBC-generated oxidative stress could have a major role in the oxidative and inflammatory cascade involved in both sickle cell disease/trait and atherosclerosis.

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# **Chapter VIII**

## **Publications & Communications**



## VIII. Publications & Communications

### VIII.1 Articles related to my published in Peer-reviewed scientific Journals

1. **Chirico EN** and Pialoux V. (2012) Role of oxidative stress in the pathogenesis of sickle cell disease. *IUBMB Life*. 64, 72-80.
2. **Chirico E**, Faes C., Martin C., Oyonno-Engelle S., Feasson L., Canet-Soulas E., Dubouchaud H., Messonnier L.; Pialoux V. (2012) Exercise training blunts oxidative stress in sickle cell trait carriers. *J Appl Physiol*. 112, 1445-53
  - Article selected for an editorial in JAP; 2012: 112 (9): 1443:1444
3. Aufradet E., Bessaad A., Alsaïd H., Schäfer F., Sigovan M., De Souza G., **Chirico E**, Canet-Soulas E., and Martin C. (2012) In vivo cardiac anatomical and functional effects of wheel running in mice by magnetic resonance imaging, *Exp. Biol. Med. (Maywood)* 237, 263–270.
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5. Chirico E., Chauveau F., Geloën, A., Patsouris D., Vidal H., Rieusset J., Pialoux V., and Canet-Soulas E. MRI evidence of exercise-induced improvement of oxidative stress and inflammation in the brain of old ApoE<sup>-/-</sup> mice. *Submitted in Circulation*.

## VIII.2 International Conference Proceedings

1. **Chirico EN.**, Faes C., Messonnier L., Féasson L., Oyonno-Enguelle S., Martin C. and Pialoux V. *Exercise Training Blunts Oxidative Stress in Sickle Cell Trait Carriers*. VIème congrès Européen sur la drépanocytose. Strasbourg, France. April 2011.
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4. **Chirico EN.**, Patsouris D., Geloën A., Rieusset J., Pialoux V., Canet-Soulas E. The role of exercise on oxidative stress and inflammation in macrophages involved in atherosclerosis. Experimental Biology. San Diego, CA. March 2012.
  - 2012 International Early Career Physiologist Travel Award recipient
5. **Chirico EN.**, Fabien Chauveau, Geloën A., Rieusset J., Canet-Soulas E., Pialoux V. The role of exercise on oxidative stress and inflammation in the aging brain. Experimental Biology. San Diego, CA. March 2012.
  - 2012 International Early Career Physiologist Travel Award recipient
6. **Chirico EN.**, Patsouris D., Geloën A., Rieusset J., Pialoux V., Canet-Soulas E. The role of exercise and a high fat/high cholesterol diet on oxidative stress and inflammation in the progression of atherosclerotic plaque development. European Congress on Obesity. Lyon, France. May 2012
7. **Chirico EN.**, Chauveau F, Patsouris D., Geloën A., Rieusset J., Pialoux V., Canet-Soulas E. The role of exercise on oxidative stress and inflammation in macrophages involved in atherosclerosis. European Atherosclerosis Society. Milan, Italy May 2012.
  - EAS Travel Award recipient
8. **Chirico EN.**, Chauveau F, Patsouris D., Geloën A., Rieusset J., Pialoux V., Canet-Soulas E. Inflammation and macrophage imaging in the aging ApoE<sup>-/-</sup> mouse: the impact of exercise. World Molecular Imaging Conference. Dublin, Ireland. September 2012
  - WMIC Student Travel Award recipient
  - Presentation nominated for poster award

# Chapter IX

## Annex

## **IX. Annex**

### **IX.1 Published Papers**

#### **IX.1a Role of Oxidative Stress in the Pathogenesis of Sickle Cell Disease**

## Critical Review

# Role of Oxidative Stress in the Pathogenesis of Sickle Cell Disease

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### Summary

Sickle cell disease (SCD) is a class of hemoglobinopathy in humans, which causes a disruption of the normal activities in different systems. Although this disease begins with the polymerization of red blood cells during its deoxygenating phase, it can erupt into a cascade of debilitating conditions such as ischemia-reperfusion injury, inflammation, and painful vaso-occlusion crises. The purpose of this review is to discuss how these phenomena can result in the formation of oxidative stress as well as limit nitric oxide (NO) bioavailability and decrease antioxidant status. The cumulative effects of these traits cause an increase in other forms of reactive oxygen species (ROS), which in turn intensify the symptoms of SCD and generate a vicious circle. Finally, we will discuss antioxidant therapeutic strategies that limit ROS generation and subsequently increase NO bioavailability with respect to endothelial protection in SCD. © 2011 IUBMB

IUBMB *Life*, 64(1): 72–80, 2012

**Keywords** nitric oxide; reactive oxygen species; hemoglobin; antioxidants.

**Abbreviations** ACS, acute chest syndrome; A-HOOH, ascorbic acid, vitamin C; A-OO, dehydroascorbate; BH<sub>4</sub>, tetrahydrobiopterin; eNOS, endothelial nitric oxide synthase; GPX, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione, glutathione disulfide; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; HbA, hemoglobin A; HbS, hemoglobin S; ICAM-1, intracellular adhesion molecule-1; NF-κB, nuclear factor-κB; NO, nitric oxide; O<sub>2</sub><sup>•−</sup>, superoxide; •OH, hydroxyl radical; ONOO<sup>−</sup>, peroxynitrite; PHT, pulmonary hypertension; RBC, red blood cell; ROS, reactive oxygen species; SCD, sickle cell disease; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1; XO, xanthine oxidase

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### INTRODUCTION

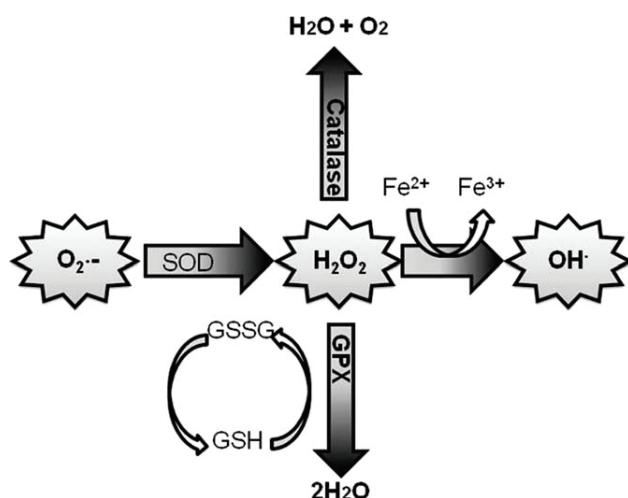
Sickle cell disease (SCD) is a class of hemoglobinopathy, which results from a single mutation in the β-globin chain inducing the substitution of valine for glutamic acid at the sixth amino acid position (1). This mutation leads to the production of abnormal hemoglobin (hemoglobin S [HbS]). In addition to homozygous sickle cell disease (HbSS), other forms such as HbSC and HbSβ-thalassemia also exist.

The pathogenesis of SCD occurs due to the polymerization of deoxygenated HbS. This polymer formation alters the normal biconcave disc shape into a rigid, irregular shaped, unstable cell (1), causing intravascular hemolysis to release hemoglobin into the plasma (2). The repeated polymerization can lead to a cyclic cascade inciting blood cell adhesion, vaso-occlusion, and ischemia-reperfusion injury. These independent manifestations can interact by altering the levels of reactive oxygen species (ROS) and antioxidants. The combination of these actions is associated with inflammatory responses in many organs and may produce secondary disease states such as acute chest syndrome (ACS), pulmonary hypertension (PHT), and, indirectly, stroke.

### OXIDATIVE STRESS

Molecular oxygen has the ability to form highly reactive metabolites such as superoxide anion radical (O<sub>2</sub><sup>•−</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and hydroxyl radical (•OH). These reduced metabolites of oxygen are referred to as “reactive oxygen species” (Fig. 1). ROS can induce oxidative damage to the cell and can form a very stable structure by extracting electrons from other sources. ROS is also able to generate other forms of ROS. Superoxide can be dismutated into H<sub>2</sub>O<sub>2</sub> and oxygen. H<sub>2</sub>O<sub>2</sub> has the ability to form the more damaging •OH, through a combination of the Fenton and Haber-Weiss reactions (3)

The inhibition of the mitochondrial electron transport chain activity can lead to ROS generation by inducing a leak of electrons from complex I (4). Generation of ROS can also result from the activation of enzymes such as xanthine oxidase (XO), NADPH oxidase, nitric oxide synthase (NOS), cytochrome



**Figure 1.** Balance of ROS and antioxidants. Oxidative stress is the imbalance between the production of ROS and antioxidants. The antioxidant properties of GPX, SOD, and catalase control the production of oxygen species. Abbreviations: GPX, glutathione peroxidase; GSH, reduced glutathione; GSSG, glutathione disulfide;  $\text{H}_2\text{O}_2$ , hydrogen peroxide;  $\text{O}_2^{\bullet-}$ , superoxide;  $\text{OH}^\bullet$ , hydroxyl radical; ROS, reactive oxygen species; SOD, superoxide dismutase.

P450, cyclo-oxygenase, and lipoxygenase. All these enzymes can be activated during the repeated cycle of hypoxia/reoxygenation or ischemia/reperfusion (5, 6).

Major ROS defense mechanisms include enzymatic and non-enzymatic systems. These protective mechanisms include the enzymatic antioxidants: superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX), and nitric oxide (NO) and the nonenzymatic antioxidants: tocopherols, reduced glutathione (GSH), carotenoids, ascorbic acid (A-HOOH), lipoic acid, ubiquinol, selenium, riboflavin, zinc, carotenoids, and uric acid as well as metal-binding proteins. The ROS which are not neutralized, can target biological molecules such as DNA, lipids, proteins, and carbohydrates, which can result in cell dysfunction or cell death.

## GENERATION OF ROS IN SCD

SCD causes harmful pathophysiological effects, including sickling, vaso-occlusion, and ischemia-reperfusion injury. These complications have a cyclic nature which involve the generation and impairment of oxidative stress. Studies have shown relationships between markers of oxidative stress and common secondary diseases in SCD, such as ACS and PHT (7, 8). Although there is elevated oxidative stress in SCD, these markers can also be mediated by other outside factors such as diet, physical activity, environment, and other comorbid diseases associated

with SCD. Therefore, the use of oxidative stress as a potential marker of disease severity has yet to be fully studied.

## Blood Cell Auto-Oxidation

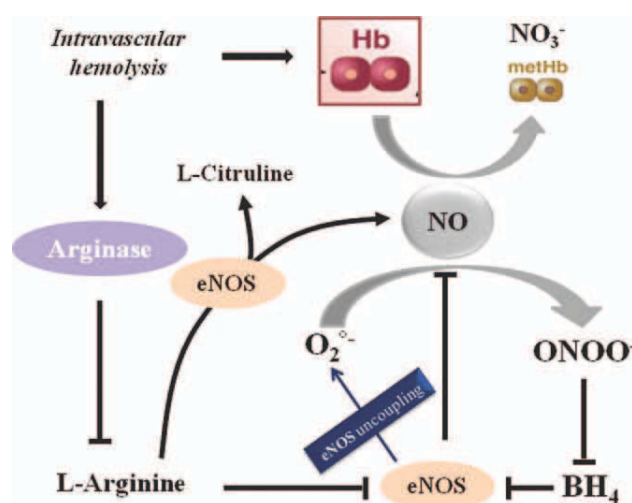
The intracellular polymerization of HbS during deoxygenation is the primary pathogenetic event in SCD. Polymerization can transform a normal red blood cell (RBC) into a dense, inflexible blood cell. The rate of polymerization has been shown *in vitro* to be correlated with the concentration of HbS and with the cell-free heme released after autooxidation (9, 10).

The RBC reoxygenation phase is a major source of free radical production in SCD. During this period, normal RBCs can generate a significant amount of superoxide due to an electron transfer between the heme iron and oxygen. In the presence of oxygen, heme auto-oxidizes inducing methemoglobin and superoxide formation. Although both hemoglobin A (HbA) and HbS blood have a tendency to autoxidize into methemoglobin and superoxide (11, 12), some studies show that HbS can auto-oxidize 1.7 times faster than HbA (13, 14), while others show a comparable rate (15). Unlike HbA, which can counter this reaction to form harmless byproducts, HbS can become overwhelmed by the continual source of superoxide and, via its dismutation,  $\text{H}_2\text{O}_2$  (11). The formation of  $\text{H}_2\text{O}_2$ , when exposed to methemoglobin, decomposes hemoglobin and releases iron. This iron can then react with remaining  $\text{H}_2\text{O}_2$  to further produce  $\text{OH}^\bullet$ , the most reactive and harmful of the reactive species (11). Sick cells ultimately generate about twofold greater quantities of superoxide,  $\text{H}_2\text{O}_2$ , and  $\text{OH}^\bullet$  than HbA (16).

## Sickling and Hemolysis

The sickle RBC often ruptures during its transport through the blood vessels. Hemolysis, along with the consequence of repeated sickling and unsickling, causes the premature destruction of erythrocytes (12). Studies have shown that sickle erythrocytes have a decreased half-life (17), only surviving for 10 days (17) compared with 50 days in normal RBCs (13). Because of their short half-life, there is a rapid turnover of RBCs, resulting in an increased proportion of reticulocytes. Present in these young cells is a high concentration of arginase, which can also be released into the plasma during hemolysis (18). About one-third of the destruction occurs within the vessels, releasing several grams of hemoglobin and iron (13) into the plasma.

SCD patients have higher levels of hemolysis than healthy subjects, although the only true marker of hemolysis is actual measurement of the blood cell lifespan. Uncorrected reticulocyte count (i.e., reticulocytes expressed in percentage), as a measure of RBC turnover, is a secondary option (19). In a study looking at SCD patients in comparison with healthy volunteers, it was found that plasma arginase concentration, a biomarker for hemolysis, was significantly higher in SCD patients and correlated strongly with the concentration of plasma-free hemoglobin (8). Other clinically used markers such as bilirubin and lac-



**Figure 2.** Degradation of NO. NO is decreased in three ways: through the reaction with  $O_2^{\bullet-}$  forming  $ONOO^-$  and through its inhibition via the byproducts of hemolysis. Abbreviations: BH<sub>4</sub>, tetrahydrobiopterin; eNOS, endothelial nitric oxide synthase; NO, nitric oxide;  $O_2^{\bullet-}$ , superoxide;  $ONOO^-$ , peroxynitrite. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

tate dehydrogenase are a less accurate measure (19). This increase in the rate of hemolysis is a starting point for many of the subsequent complications of SCD, especially considering its involvement with NO.

### Fate of NO

NO is an important regulator of vascular tone, blood flow, and adhesion. In SCD patients, not only is the generation NO dramatically decreased, but the amount available for use is limited as well. Its bioavailability can be reduced in several ways: 1) via increases in the free radical superoxide, 2) through the products of hemolysis (free heme and arginase), and 3) through the “uncoupling” of endothelial NOS (eNOS; Fig. 2).

The interaction between superoxide and NO is more detrimental than their individual actions. While in normal systems superoxide can easily be dismutated by SOD, the overproduction of  $O_2^{\bullet-}$  seen in SCD overwhelms the defenses of body and reacts at diffusion-limited rates with NO. The reaction of NO and superoxide results in the generation of peroxynitrite ( $ONOO^-$ ): a powerful and highly reactant oxidant. The generation of  $ONOO^-$  is favored over spontaneous superoxide dismutation and NO autooxidation; the interaction is faster than both NO's reaction with hemoglobin and superoxide's reaction with SOD (11).  $ONOO^-$  has the opportunity to form  $\bullet OH$  and nitrogen dioxide ( $NO_2$ ), two other potent oxidants (11). The reaction between NO and  $O_2^{\bullet-}$  is double in that it not only further decreases the concentration of NO, but also produces more reactive free radicals.

Cell-free hemoglobin has a large impact on the bioavailability of NO, resulting in endothelial dysfunction and vasoconstriction.

Hemolysis increases the concentration of plasma hemoglobin, which allows for the reaction of both deoxy-HbS and oxy-HbS with NO. The increased propensity for this reaction leads to the decrease in NO bioavailability. The binding of NO to deoxygenated hemoglobin results in the formation of a stable  $Fe^{2+}Hb-NO$  complex, which can readily react in Fenton reactions (2). The reaction between NO and oxygenated hemoglobin can form methemoglobin and nitrate. This becomes problematic in SCD because it has been reported that these patients have a much higher level of cell-free hemoglobin and methemoglobin leading to the consumption and decreased bioavailability of NO (18, 20–23). It has been shown that SCD patients have up to 20  $\mu M$  of heme in blood compared with the normal level of 0.2  $\mu M$ . During episodes of crisis, this number can increase several fold (21). A study by Reiter and Gladwin (20) found that in SCD patients, those with plasma heme concentrations of 6  $\mu M$  or greater had a decreased NO response by 80%. Even with levels as low as 1  $\mu M$ , the amount of NO able to reach smooth muscle cells was significantly reduced, concluding that during crisis plasma-free hemoglobin levels were high enough to sufficiently deplete NO bioavailability (21). The effect of NO scavenging via cell-free hemoglobin has a role in increasing vasoconstriction and decreased oxygen delivery through the abatement of NO-dependent vasodilation (24).

NO is further reduced through the consequence of eNOS “uncoupling.” eNOS is an enzyme made up of a reductase domain and an oxygenase domain which produces NO. Normally, through the catalytic action of eNOS, tetrahydrobiopterin (BH<sub>4</sub>) transfers an electron in the oxygenase domain, converting L-arginine into NO and L-citrulline. However, under certain conditions, eNOS can produce superoxide rather than NO. The two main cofactors of this mechanism, L-arginine and BH<sub>4</sub>, are reduced due to previous repercussions of SCD, therefore, generating superoxide (22, 25).

L-Arginine concentrations are decreased in SCD, and because it is the rate-limiting substance in NOS, it can uncouple eNOS and generate additional superoxide (26). Arginine is considered a major target in hemolysis because its concentration can be diminished by arginase, one of the products of hemolysis. Arginase can out-compete the actions of eNOS by converting L-arginine into ornithine and urea. A deficiency of L-arginine interrupts the electron flow through the eNOS domains and favors the generation of superoxide over NO (25). In some SCD patients, L-arginine levels are significantly decreased (22), coinciding with an almost twofold increase in arginase levels (18, 22, 27), resulting in a significant decrease in NO bioavailability. The important role of L-arginine in NO production has been demonstrated with the supplementation of L-arginine in the diet of both mice and humans. This treatment was shown to reverse NO resistance (28), increase NO bioavailability, and increase antioxidant activity (29). These studies demonstrate a tight association between the decrease in arginine due to hemolysis and NO levels.

Another important cofactor of eNOS, BH<sub>4</sub>, is also affected by the repercussions of SCD, leading to superoxide generation.



A deficiency of BH<sub>4</sub> can lead to the production of superoxide via the “uncoupling” of eNOS (25). BH<sub>4</sub> can be inactivated by ONOO<sup>−</sup> (25, 30), which is over abundantly produced in SCD, via the reaction between NO and O<sub>2</sub><sup>•−</sup>. In this situation, similar to L-arginine, eNOS functions as a producer of superoxide rather than NO (25). *In vitro*, human, and animal studies have demonstrated the link between BH<sub>4</sub>, ONOO<sup>−</sup>, and superoxide in eNOS-producing NO. In cultured endothelial cells, a deficiency of BH<sub>4</sub> was shown to uncouple eNOS, while its supplementation completely restored eNOS activity (31) and increased NO production (31). The BH<sub>4</sub>-reducing capabilities of ONOO<sup>−</sup> was further demonstrated in human, where ONOO<sup>−</sup> increased 8-F2-isoprostanes (32) and supplementation of BH<sub>4</sub> recoupled eNOS, increased NO production, and inhibited superoxide (31). Sickie transgenic mice demonstrated a decrease in BH<sub>4</sub> levels responsible for eNOS uncoupling (25). ONOO<sup>−</sup> increased the production of O<sub>2</sub><sup>•−</sup> in the vessels of control mice in contrast to eNOS deficient mice, while increasing aortic lipid peroxides in rats (32). The combined effects of these studies demonstrate the vital role of BH<sub>4</sub> in assisting eNOS function.

### **RBC Adhesion and Vaso-Occlusion**

In addition to the decreased bioavailability of NO, which can reduce vasodilation, a variety of adhesion molecules expressed on sickled erythrocytes can also impair blood flow. Under normal circumstances, circulating blood cells loosely come into continuous contact with the endothelial cells of blood vessels. In conditions, such as SCD, there is an increase in adherence to the vessel walls. Activation of vascular endothelial cells and circulating blood cells represent the continual inflammation seen in SCD. Upon activation, circulating white blood cells and platelets express adhesion glycoproteins. Consequently, endothelial dysfunction is modulated by the interaction between blood cells and platelets and the cellular and molecular components in the endothelium. In this context, blood cell adherence to the endothelium can be modulated by factors such as decreased NO bioavailability, hemolysis, ROS, and inflammation. This abnormal interaction involves adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), E-selectin, and P-selectin, which are overexpressed in SCD (25, 33–36). Because of this overexpression, sickled RBCs are at least 2.5 times more likely to adhere to endothelial cells than normal RBCs (34, 37). VCAM-1 and ICAM-1 are associated with decreased NO bioavailability (33–35) and increased hemolysis (27). Levels of adhesion correlate positively with lactate dehydrogenase (33) and arginase (22), often used markers of hemolysis (27). In both human and cross-species studies, sickled RBCs incubated with a NO donor decreased adhesion (34). These results suggest that a decrease in NO bioavailability and an increase in hemolysis can assist in furthering blood cell adherence.

Oxidative stress can promote sickled blood cell adherence to the endothelium, while the supplementation of antioxidants can

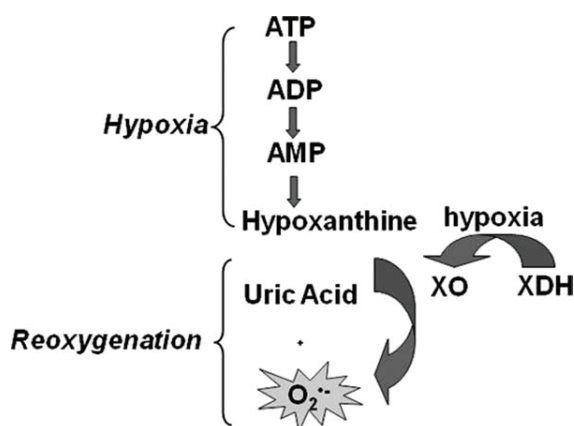
reduce the expression of adhesion molecules. The interaction between sickled RBCs and endothelial cells is associated with a threefold increase in oxidative stress, as measured by thiobarbituric acid reactive substances (TBARS) (37). The increase in TBARS was inhibited by the addition of SOD and catalase, which led to a 50% decrease in lipid formation (37). Additions of the antioxidant *N*-acetylcysteine (NAC) inhibited the expression of ICAM-1 induced by H<sub>2</sub>O<sub>2</sub> (38), while the addition of a different antioxidant pyrrolidine dithiocarbamate inhibited the expression of VCAM-1 (39). The increase in oxidative stress is linked to the activation of nuclear factor-κB (NF-κB) (37) as its expression is increased during periods of adhesion (37). NF-κB can alter the gene expression of VCAM-1, ICAM-1, and E-selectin, leading to their increase (40). An inhibitor of NF-κB (sulfasalazine) has been observed to decrease endothelial cell expression of VCAM-1, ICAM-1, and E-selectin in both humans and mice (36). NF-κB can also be regulated by tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). These inflammatory cytokines are raised in SCD patients and can influence the expression of adhesion molecules as well (33). In both human and cross-species studies, normal cells enhanced with TNF-α increased adhesion, while the use of a NO donor abrogated this increase (34). Lipolate downregulated the TNF-α-induced expression of ICAM-1 and VCAM-1 (41). The link between the expression of cytokines and oxidative stress is further verified through the addition of antioxidants. The inhibition of NF-κB induced adhesion via antioxidants demonstrates the interaction of oxidative stress on adhesion.

Adhesion of sickled RBC to the endothelium and other blood cells, as well as precapillary obstruction by rigid, sickled blood cells can initiate vaso-occlusion in the microvasculature (42). This results in vaso-occlusion crises, which include recurrent episodes of severe pain, in patients with SCD. The frequency of vaso-occlusive episodes was reduced 5-fold due to the administration of NAC (43), which was additionally shown to inhibit ICAM-1 expression (38). The vessel occlusion causes tissue ischemia in downstream areas deprived of oxygen. Eventually these venules reestablish blood flow, leading to reperfusion injury (44).

### **Hypoxia/Reperfusion Injury**

A common repercussion of SCD due to adhesion and vaso-occlusion is the occurrence of hypoxia. The cessation of blood flow to tissues causes an ischemic or hypoxic environment. During this state, the limited concentration of oxygen available to tissues results in an inadequate amount of nutrients delivered to support the metabolic needs. ROS can be generated at various points during this undesirable environment: during hypoxia in both the mitochondria and in tissues, as well as during the reperfusion phase that follows. This phase can cause reperfusion injury, referring to the damage caused to the vessel and tissue when oxygen is reintroduced to the tissues, leading to an increase in the concentration of radical species (45).





**Figure 3.** Hypoxia/reoxygenation phenomenon. Under conditions of hypoxia, hypoxanthine and XO are generated. During reoxygenation, these two products can be converted into superoxide. Abbreviation:  $O_2^{\bullet-}$ , superoxide.

Under hypoxic conditions, as seen in SCD, adenosine triphosphate (ATP) is consumed to adenosine diphosphate and adenosine monophosphate. If oxygen supply continues to decrease to certain levels, adenosine monophosphate is catabolized, leading to the accumulation of hypoxanthine and xanthine in the tissue (44, 45). The produced XO can then lead to deleterious effects due to the restitution of blood flow and therefore oxygen to the cells. It is when oxygen is reintroduced to these tissues ("reoxygenation") and reacts with the hypoxia-formed XO, that the damaging effects of oxidative stress are seen. This reaction results in the conversion of hypoxanthine and xanthine by XO into superoxide (40, 44, 45) (Fig. 3).



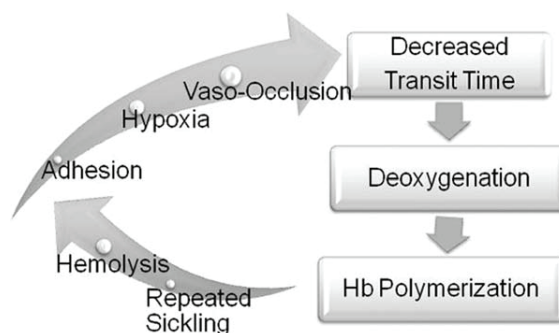
A study by Osarogiagbon et al. (44) on reperfusion injury in sickle mice found that the mice who were subjected to hypoxic conditions showed a significantly higher proportion of XO than both control mice and sickle mice in ambient conditions. As supported by the equation above, this increase in hypoxia-generated XO can lead to the production of  $O_2^{\bullet-}$ . The increase in  $O_2^{\bullet-}$  can lead to a chain reaction of free radicals as observed by the increase in ethane excretion and  $\bullet OH$ s, two markers of oxidative stress. To further verify that the increase in oxidative stress was indeed a result of XO, administration of allopurinol, an XO inhibitor, was shown to diminish ethane excretion levels (44). An increase in XO has been also associated with inflammatory cytokines such as  $TNF-\alpha$ ,  $IL-1\beta$ , and  $IFN\gamma$  in many different tissues and diseases (46–48). As discussed in the previous section, there is a strong correlation between oxidative stress and inflammatory cytokines. The cyclic nature of this disease is evident as the ROS-induced cytokines, which can cause adhesion and hypoxia, are also produced as a result of hypoxia-induced  $O_2$ -production.

Hypoxia causes an increased proportion in the amount of sickled cells (44), possibly due to the sickle-inducing extended deoxygenation period brought about by the decreased oxygen (49). Conditions of hypoxia can also further increase the adhesive interactions within the vessel walls, exacerbating vaso-occlusion. Setty and Stuart demonstrated that sickled RBC, contrary to normal cells, exposed to hypoxia have a 66% greater risk of adhering to both the macrovasculature (as seen in the aorta) and the microvasculature (as seen in the retina) (35). It was shown that hypoxia regulates the production of VCAM-1 and ICAM-1, although VCAM-1 is solely responsible for the adherence of sickled cells to the endothelium (35). This regulation could be due to NF-kB, which is abundantly observed after hypoxia (44, 50), and can alter the expression of these adhesion molecules (Figs. 4 and 5).

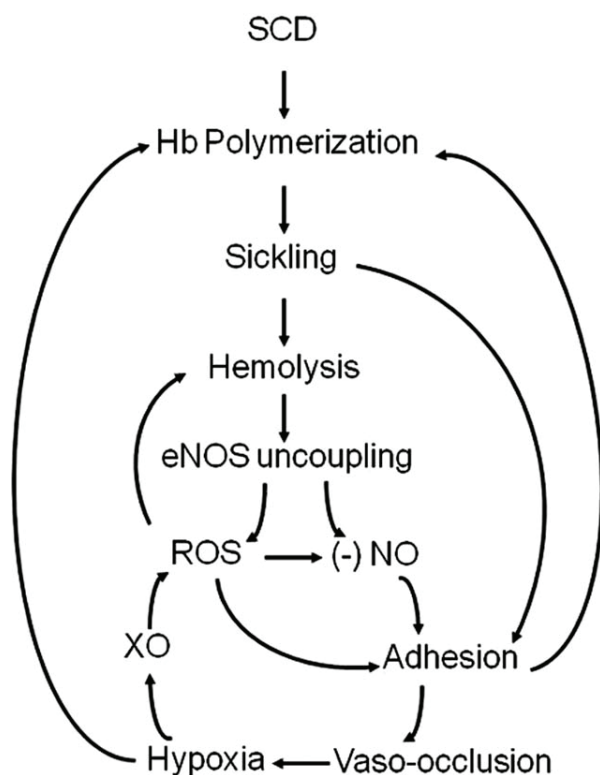
### Antioxidants

In addition to the oxygen species that are formed in SCD, the protective mechanisms such as antioxidants are decreased. Those that provide enzymatic defense, including SOD, GPX, catalase, and heme oxygnease-1, and those that scavenge free radicals, such as glutathione, vitamin C, and vitamin E, are most affected (51).

SOD has been shown to increase in some studies of SCD (52, 53), while others show decreased levels (29, 54). A study by Das and Nair (53) demonstrated an increase in SOD in RBCs of SCD patients concluding that this may be a defense mechanism due to the increase in oxidative stress and could possibly cause a buildup of  $H_2O_2$ . A contrasting study by Schacter et al. (54) showed that SOD levels decreased in proportion to disease severity in HbS patients compared with healthy black volunteers. This difference was suggested to be due to a more rapid degradation of SOD, possibly from oxygen radicals. They also showed that black controls have a naturally higher level of SOD than white controls; meaning that the main



**Figure 4.** Cyclic reaction of RBC sickling. The normal oxygenation/deoxygenation process occurring in the lungs can be disturbed by an increase in adhesion, hypoxia, or vaso-occlusion. This can lead to increased RBC sickling and hemolysis, continuing the cycle.



**Figure 5.** Vicious circle of SCD. The cyclic reaction beginning with SCD leading to the downstream effects which cycle back to restart the complications of the disease. Abbreviations: eNOS, endothelial nitric oxide synthase; Hb, hemoglobin; NO, nitric oxide;  $O_2^{\bullet-}$ , superoxide; ROS, reactive oxygen species; XO, xanthine oxidase.

discrepancy between the studies could lie in the control subjects used (54).

Under normal circumstances,  $H_2O_2$ , which is either produced via a two electron transfer or due to sickling, is removed by two methods: GPX or catalase. Due to the decreased levels of these enzymes in SCD patients (8, 11, 53), the endogenously produced  $H_2O_2$  cannot be sufficiently cleared (11).

GSH, a cofactor for GPX, is easily oxidized to glutathione disulfide (GSSG) through its reduction of free radicals and ROS and is an essential element for GPX to reduce  $H_2O_2$ . GSH concentration is significantly reduced in sickle cell patients (16, 29), with some studies noting a 50% decrease in HbS compared with HbA (55). GPX concentrations are also reduced (51), with a direct relationship to the severity of the disease (29). A study by Morris et al. (8) found that SCD patients had significantly lower concentration of total glutathione (GSH + GSSG) in the plasma and within the RBC compared with healthy patients. These results suggest that the overabundance of oxidative stress leads to consumption or inactivation of these protective elements.

There is a discrepancy between studies on the levels of catalase in SCD; a study from Dasgupta et al. (29) has shown decreased levels, whereas another study by Das and Nair (53) has observed increased levels in patients. The increase in catalase could be a protective effect to scavenge  $H_2O_2$ , while the decreased levels could be due to the overwhelming concentration of oxidative stress.

Heme oxygenase-1 (HO-1) is a cytoprotective enzyme that is activated in response to heme-induced oxidative stress. HO-1 can inhibit NF- $\kappa$ B, VCAM-1, ICAM-1, leukocyte-endothelial interactions, and hypoxia-reperfusion induced stasis. In addition, the byproducts of HO-1, including carbon monoxide, biliverdin and iron, have antioxidant and anti-inflammatory properties. Although humans and transgenic mice have been shown to up-regulate HO-1 in response to heme-induced oxidative stress, SCD patients have insufficient activity to handle the burden of heme (2). SCD patients are unable to activate HO-1 in response to prolonged hemolysis, preventing the inhibition of oxidative stress, vaso-occlusion, and hypoxia/reperfusion injury (2).

### Antioxidant Therapeutic Strategies

Several treatment studies have been shown to be effective to reduce pathological consequences of the disease (see Wood et al. (13) and Nur et al. (56) for review). Susceptibility to peroxidation, due to an increase in ROS or during conditions of reduced oxygen, can increase threefold in sickle cell patients. *In vitro*, preincubation of sickle erythrocytes with vitamin E decreased the susceptibility to peroxidation. This finding is maintained *in vivo*, where those with a vitamin E deficiency increased levels of peroxidation compared with control subjects (57). A study evaluating the effects of SCD by Natta et al. (58) found that after only 10 weeks of vitamin E supplementation, the percentage of irreversibly sickled red cells decreased from 25% to 11%. A decrease in sickled RBC was also observed during a supplementation period with vitamin E possibly due to the almost twofold decrease of ROS and the 1.2-fold increase in the concentration of GSH (16).

The additions of A-HOOH and dehydroascorbic acid supplements were able to inhibit dense RBC formation and lipid peroxidation levels in sickle cell patients (59). Another study by Amer et al. (16) found that supplementation of vitamin C helped to decrease ROS production almost fourfold while increasing the concentration of GSH almost twofold. It may be through this mechanism that A-HOOH supplements were shown to prevent  $H_2O_2$ -induced hemolysis (16).

Finally, *in vitro* supplementation of alpha-lipoic acid, known to have potent antioxidant properties, can inhibit RBC sickling by 50% (59), decrease oxidation (60), protect peroxyl radical-induced hemolysis, and increase GSH synthesis (61).

Taken together these promising results should encourage the development of such antioxidant therapy. However, the conclusions of clinical trials using antioxidant therapeutic treatment in SCD are not so enthusiastic (25). Wood et al. (25) suggest that the SCD-induced ROS generation may greatly overwhelm the

capacity of the exogenous antioxidants, leading to the conclusion that a cocktail of multiple antioxidants may be more efficient (59).

Therapeutic strategies that focus on decreasing ROS production, instead of increasing their neutralization, were also recently studied. Hydroxyurea (HU) can reduce the occurrence of vaso-occlusive crises and pulmonary events by inducing fetal hemoglobin. However, in addition to fetal hemoglobin, HU can limit ROS production and NO scavenging via a decrease in hemolysis. To determine mechanisms, *in vitro* studies have found a decrease in adhesion between HbS RBC and the endothelium, as well as a decrease in adhesion molecules on sickle cell reticulocytes (62). Some studies have suggested that HU induces a NO response (63), as NO is well documented in the regulation of adhesion. Because of the importance in maintaining proper NO levels, exogenous NO treatment is often beneficial. Inhaled NO was shown to increase plasma NOx in some case studies, and decrease adhesion and ischemia/reperfusion injury in animal studies (13). Arginine therapy is shown to have a positive effect on vasodilation, possibly through the reduction of oxidative stress and hemolysis. Kaul et al. (28) suggested that arginine therapy in SCD mice prevented the oxidative stress-induced hemolysis of RBC. An increase in the antioxidant GSH and enhanced NO production was also observed in these mice, possibly due to a reversal of eNOS uncoupling (28).

Iron chelator desferoxamine (64), a catalase mimetic (40) and an NF- $\kappa$ B inhibitor (65) were shown to attenuate oxidative stress, adhesion, and inflammation in murine models of SCD. Although not tested yet in SCD, the use of SOD mimetic (such as tempol) may potentially be beneficial to target the anion superoxide clearly identified in the pathogenesis of SCD (25).

## CONCLUSION

SCD is a complicated disease in which there is no cause and effect consequence, yet rather a continuous cycle which further perpetuates the disease. The production of ROS results in hemolysis, endothelial dysfunction, cell adhesion, and vaso-occlusion. Within each of these complications is the opportunity to produce more ROS, further exacerbating the disease. These complications often double as risk factors for vascular diseases, such as PHT and ACS, leads to a decreased lifespan and poor quality of life. The use of antioxidant supplementation has shown some reduction in the detrimental effects of oxidative stress seen in SCD. However, future research should focus on a combination of antioxidant supplements or on drug treatments that specifically target ROS at its production site. By limiting the production of ROS, many of the complications of SCD could be diminished.

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### **IX.1b Exercise training blunts oxidative stress in sickle cell trait carriers**

## Exercise training blunts oxidative stress in sickle cell trait carriers

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**Chirico EN, Martin C, Faës C, Féasson L, Oyono-Enguélé S, Aufradet E, Dubouchaud H, Francina A, Canet-Soulas E, Thiriet P, Messonnier L, Pialoux V.** Exercise training blunts oxidative stress in sickle cell trait carriers. *J Appl Physiol* 112: 1445–1453, 2012. First published February 9, 2012; doi:10.1152/jappphysiol.01452.2011.—The aim of this study was to analyze the effects of exercise training on oxidative stress in sickle cell trait carriers. Plasma levels of oxidative stress [advanced oxidation protein products (AOPP), protein carbonyl, malondialdehyde (MDA), and nitrotyrosine], antioxidant markers [catalase, glutathione peroxidase (GPX), and superoxide dismutase (SOD)], and nitrite and nitrate (NOx) were assessed at baseline, immediately following a maximal exercise test ( $T_{ex}$ ), and during recovery ( $T_{1h}$ ,  $T_{2h}$ ,  $T_{24h}$ ) in trained (T: 8 h/wk minimum) and untrained (U: no regular physical activity) sickle cell trait (SCT) carriers or control (CON) subjects (T-SCT,  $n = 10$ ; U-SCT,  $n = 8$ ; T-CON,  $n = 11$ ; and U-CON,  $n = 11$ ; age:  $23.5 \pm 2.2$  yr). The trained subjects had higher SOD activities ( $7.6 \pm 5.4$  vs.  $5.2 \pm 2.1$  U/ml,  $P = 0.016$ ) and lower levels of AOPP ( $142 \pm 102$  vs.  $177 \pm 102$   $\mu$ M,  $P = 0.028$ ) and protein carbonyl ( $82.1 \pm 26.0$  vs.  $107.3 \pm 30.6$  nm/ml,  $P = 0.010$ ) than the untrained subjects in response to exercise. In response to exercise, U-SCT had a higher level of AOPP ( $224 \pm 130$  vs.  $174 \pm 121$   $\mu$ M,  $P = 0.012$ ), nitrotyrosine ( $127 \pm 29.1$  vs.  $70.6 \pm 46.6$  nM,  $P = 0.003$ ), and protein carbonyl ( $114 \pm 34.0$  vs.  $86.9 \pm 26.8$  nm/ml,  $P = 0.006$ ) compared with T-SCT. T-SCT had a higher SOD activity ( $8.50 \pm 7.2$  vs.  $4.30 \pm 2.5$  U/ml,  $P = 0.002$ ) and NOx ( $28.8 \pm 11.4$  vs.  $14.6 \pm 7.0$   $\mu$ mol·l<sup>-1</sup>·min<sup>-1</sup>,  $P = 0.003$ ) in response to exercise than U-SCT. Our data indicate that the overall oxidative stress and nitric oxide response is improved in exercise-trained SCT carriers compared with their untrained counterparts. These results suggest that physical activity could be a viable method of controlling the oxidative stress. This could have a beneficial impact because of its involvement in endothelial dysfunction and subsequent vascular impairment in hemoglobin S carriers.

antioxidants; physical activity; hemoglobin

SICKLE CELL DISEASE (SCD) is a hemoglobinopathy resulting from a single mutation in the  $\beta$ -globin chain gene, inducing the substitution of valine for glutamic acid at the sixth amino acid position. This mutation leads to the production of abnormal hemoglobin (HbS). The pathogenesis of SCD occurs due to the polymerization of deoxygenated HbS, eventually leading to the rigidity and sickling of red blood cells (RBC). The

most deleterious pathophysiological effects of sickling include endothelial dysfunction, inflammation, and vaso-occlusion (65). All these events can be traced back to an increase in oxidative stress, defined as a damaging imbalance between the production of oxidants and antioxidants (63, 66). Endothelial dysfunction, which can cause increased adhesion in the vessels leading to vaso-occlusion, hypoxia, and hemolysis, is notably due to the impairment of nitric oxide bioavailability (1, 35). Vaso-occlusion and hypoxia can generate superoxide, whereas hemolysis can inhibit NO production while generating more reactive oxygen species (66).

Subjects who present both normal [hemoglobin A (HbA)] and sickled hemoglobin (HbS) are identified as sickle cell trait (SCT) carriers. SCT is usually considered to be a benign and asymptomatic condition (20). However, several authors suggest that SCT should be reclassified as a disease state (9, 10, 32). In fact, SCT has been linked as a cofactor for morbidity and mortality (32, 60, 69) due to complications at rest (69) and during exercise (30, 31, 34) particularly in hypoxic conditions (31). An increasing number of studies have reported exercise-related sudden deaths in SCT carriers (14, 20, 31, 34). The high incidence of exercise-related deaths in SCT could be a result of RBC abnormalities, such as decreased RBC deformability (32, 68) and endothelial damage (69), associated with an increase in oxidative stress (13, 23). This hypothesis is supported by the fact that 1) RBC sickling may increase during exercise in SCT carriers (6), 2) sickle erythrocytes overproduce reactive oxygen species (ROS) (13, 23), 3) SCT carriers increase RBC oxidative stress during exercise (12), and 4) ROS induce a cascade of events including endothelial dysfunction and adhesion potentially leading to vascular occlusion (1). Taken together, these results lead to the hypothesis that oxidative stress could be involved in exercise-related complications through the vascular dysfunction mechanism seen in SCT.

In various situations, exercise training has been shown to decrease oxidative stress through an upregulation in the antioxidant system, thereby halting the overproduction of oxidants (28, 53–55). In turn, this may improve cardiovascular function by reducing endothelial dysfunction, inflammation, and adhesion (24, 35). A recent paper by Aufradet et al. (3) demonstrated that the increased endothelial activation commonly occurring in SCT (47, 48) was attenuated in trained SCT carriers compared with the untrained carriers. This effect on adhesion molecules, which are regulated by nitric oxide and stimulated by ROS (33), suggests that a training effect could be

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under the control of oxidative stress. However, to our knowledge there are no studies dealing with the effects of regular exercise training on oxidative stress and nitric oxide metabolism in SCT carriers.

The aim of the present study is to test the hypothesis that regular training improves NO metabolism and decreases oxidative stress, adhesion, and endothelial dysfunction in SCT subjects.

## METHODS

### Ethical Approval

The protocol was approved by the local ethics committee of Cameroon and was in accordance with the guidelines set by the Declaration of Helsinki. All of the subjects were volunteers and gave their written informed consent before participating in the study.

### Subjects

Eighteen SCT carriers ( $34.5 \pm 0.8\%$  HbS) and 22 subjects with normal hemoglobin (CON) participated in the study. All subjects were male students attending the University of Yaounde II (Soa, Cameroon). SCT and CON groups were divided into two subgroups on the basis of their fitness level. The 11 CON and 8 SCT who reported no regular physical activity for the two previous years were assigned to untrained (U) subgroups (U-CON and U-SCT). The 11 CON and 10 SCT who practiced soccer on a regular basis ( $>8$  h/wk minimum for several years) were assigned to trained (T) subgroups (T-CON and T-SCT). The group categorization of trained and untrained was confirmed by fitness level measured by relative maximal aerobic power as described in the results. Subjects completed a medical examination that included height and weight measurements, as well as a venous blood test to detect SCT and HIV. Exclusion criteria included the presence of a known chronic disease (hypertension, HIV), stroke, or recent malaria episode. No previous sickle cell crisis or other incident relating to hemoglobinopathy was reported in any subject.

### Exercise Protocol

All the experiments took place at General Hospital of Yaounde (Cameroon). The subjects were asked to avoid any strenuous exercise the day before the test. All meals before and after the exercise test were provided at the hospital and water was available ad libitum to ensure proper nutrition and hydration. An incremental maximal exercise test was performed on a cycle ergometer (Monark, 818E, Stockholm, Sweden). The test began with a 5-min warm up at 30 W, followed by a work rate of 70 W that was continuously increased by 35 W every 3 min until volitional exhaustion was reached. Heart rate (HR) was collected throughout the exercise using a chest belt monitor (Polar Electro, Kempele, Finland). Maximal HR ( $HR_{max}$ ; beats/min) was considered the highest recorded heart rate during the test. Maximal aerobic power (MAP; W) was assessed by linear interpolation from the HR vs. work rate curve. Blood samples were drawn from a catheterized antecubital vein on the nondominant arm and were collected at baseline (Base), immediately at the end of the exercise test ( $T_{ex}$ ), and after 1 h, 2 h, and 24 h of recovery ( $T_{1h}$ ,  $T_{2h}$ ,  $T_{24h}$ , respectively) in EDTA tubes. The samples were used to measure levels of oxidative stress [advanced oxidation protein products (AOPP), protein carbonyl, malondialdehyde (MDA), and nitrotyrosine], antioxidant [catalase, glutathione peroxidase (GPX), and superoxide dismutase (SOD)], and NO metabolism (NOx) markers, and adhesion molecules (P-selectin and E-selectin).

### SCT Confirmation, $\alpha$ -Thalassemia, and Hematological Parameters

To test for SCT, blood samples were collected in EDTA tubes at rest and the various Hb were isolated and quantified by ion-exchange

high-performance liquid chromatography (HPLC) (Variant I, Beta Thal Short Program: Bio-Rad Laboratories, Hercules, CA). Positive test results for SCT were determined by the presence of HbS, but only if  $<50\%$  of total Hb. To test for the coexistence of  $\alpha$ -thalassemia, the technique described by Chong et al. (8) was used. The only type of  $\alpha$ -thalassemia found in some SCT carriers was the heterozygous form marked by a deletion of 3.7 kb of DNA, containing one of the two linked  $\alpha$ -globin genes ( $\alpha\alpha/\alpha^3.7$ ). No other genetic Hb abnormality was found in this population. Blood for the hematological measurements was collected at rest in EDTA tubes and was analyzed using a hematology analyzer (Abbott Cell Dyn 1800 hematology analyzer, Block Scientific, NY).

### Oxidative Stress and Antioxidant Assessment

The blood samples collected at baseline,  $T_{ex}$ ,  $T_{1h}$ ,  $T_{2h}$ , and  $T_{24h}$  were centrifuged, and the aliquoted plasma was stored at  $-80^\circ\text{C}$  until analysis. All samples were assessed within the same time period.

**Plasma AOPP.** AOPP were determined in blood plasma using the semi-automated method developed by Witko-Sarsat et al. (70), as previously described (55). AOPP were measured by spectrometry on a microplate reader (TECAN Infinite M200, Mannedorf, Switzerland) and were calibrated with a chloramine-T solution that absorbs at 340 nm in the presence of potassium iodide. The absorbance of the reaction was immediately read at 340 nm on the microplate reader against a blank containing 200  $\mu\text{l}$  of PBS. AOPP activity was expressed as micromoles per liter of chloramines-T equivalents.

**Protein carbonyl.** Measurements of protein carbonyls can be used as an index of oxidative injury. Protein carbonyls were measured by spectrophotometry at 380 nm using 2,4-dinitrophenylhydrazine according to a method of Levine et al. (43).

**Catalase.** Catalase activity in the plasma was determined by the method of Johansson and Borg (29), using hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) as a substrate and formaldehyde as a standard. Catalase activity was determined by the formation rate of formaldehyde induced by the reaction of methanol and  $\text{H}_2\text{O}_2$  using catalase as enzyme.

**GPX.** GPX in the plasma was determined by the modified method of Paglia and Valentine (52), using  $\text{H}_2\text{O}_2$  as a substrate. GPX was determined by the rate of oxidation of NADPH to  $\text{NADP}^+$  after addition of glutathione reductase (GR), reduced glutathione (GSH), and NADPH.

**MDA.** Although MDA assay shows methodological limitations (42), it is the most common lipid peroxidation marker and it is still widely used as marker of oxidative stress. Concentrations of plasma MDA were determined as thiobarbituric reactive substances by a modified method of Ohkawa et al. (51), as previously described (53).

**NOx.** The end-products of endothelium nitric oxide and NOx were measured in the plasma using reagents purchased from Sigma-Aldrich based on methods previously described (45). The sum of nitrite and nitrate in the plasma (NOx) is considered an index of nitric oxide production (23).

**Nitrotyrosine.** Concentrations of plasma nitrotyrosine, as end product of protein nitration by  $\text{ONOO}^-$ , were measured as previously described (19). Nitrotyrosine was measured using reagents purchased from Sigma-Aldrich.

**SOD.** The quantitative determination of the SOD activity was performed using the Beauchamps and Fridovich's method (5), slightly modified by Oberley and Spitz (50). SOD activity was determined by the degree of inhibition of the reaction between superoxide radicals, produced by a hypoxanthine-xanthine oxidase system, and nitroblue tetrazolium.

**Adhesion molecule assessment.** sP-selectin and sE-selectin were assessed by ELISA according to the manufacturer's instructions (Diacalone Systems, Besançon, France).



### Statistical Methods

The results are presented as mean  $\pm$  SD. Anthropometric and hematologic characteristics were compared using a two-way ANOVA with Fisher least significant difference (LSD) post hoc. The data related to oxidative stress markers and antioxidants were compared between groups using a two-way repeated-measures ANOVA with Fisher LSD post hoc. Pearson's coefficient correlations were used to determine the associations between markers of oxidative stress and NOx, nitrotyrosine and adhesion markers, respectively. Statistical significance was determined by a *P* value of  $<0.05$ . Analyses were conducted using Statistica (version 8.0, Statsoft, Tulsa, OK).

### RESULTS

#### Anthropometric and Hematologic Characteristics

Data for anthropometric, hematologic, and training measurements are detailed in Table 1. Compared with the untrained subjects, the exercise trained group had a significantly higher absolute and relative MAP ( $169 \pm 50$  vs.  $214 \pm 34$  W,  $P = 0.002$  and  $2.78 \pm 0.4$  vs.  $3.17 \pm 0.5$  W/kg,  $P = 0.006$ , for U and T, respectively). Furthermore, there is no differences in absolute or relative MAP among the U and T subgroups, i.e., between U-SCT and U-CON (nonsignificant: NS) and between T-SCT and T-CON (NS). There were no significant differences in %HbS between U-SCT and T-SCT. Whereas no significant differences appeared in age, RBC count, or maximal heart rate among the four groups, U-SCT was taller and U-CON was lighter than the other three groups ( $P < 0.05$ ). T-SCT subjects had a significantly higher platelet count than U-SCT and U-CON (see Table 1).

#### Oxidative Stress Markers at Baseline and After Maximal Exercise

**AOPP.** AOPP concentrations were not different among the four groups at baseline (NS) but were significantly higher at  $T_{ex}$  than at baseline when the four groups were pooled ( $P < 0.001$ ; Table 2). Furthermore, AOPP concentrations were significantly higher in U-SCT than in the three other groups (training  $\times$  hemoglobin crossed effect,  $P < 0.05$ ).

**MDA.** At baseline, trained subjects had higher levels of MDA than untrained subjects ( $P = 0.014$ ), whereas there were no differences between SCT carriers and CON subjects. MDA concentrations were significantly increased at  $T_{ex}$ ,  $T_{1h}$ , and  $T_{2h}$  compared with baseline in U-SCT subjects, whereas they were

decreased at  $T_{1h}$ ,  $T_{2h}$ , and  $T_{24h}$  in trained subjects (Table 2). The percentage increase from baseline was significantly higher in U-SCT than the three other groups at  $T_{ex}$ ,  $T_{1h}$ ,  $T_{2h}$ , and  $T_{24h}$  (Fig. 1).

**Protein carbonyl.** There was no difference in protein carbonyl content among groups at baseline (NS). Protein carbonyl was significantly higher in all four groups at  $T_{ex}$  compared with baseline (U-CON:  $+117\%$ ,  $P = 0.0002$ ; U-SCT:  $+124\%$ ,  $P < 0.0001$ ; T-CON:  $+77\%$ ,  $P < 0.0001$ ; T-SCT:  $+71\%$ ,  $P = 0.0005$ ). U-SCT protein carbonyl concentrations were significantly higher than in the other three groups at  $T_{ex}$  ( $P = 0.003$  vs. T-CON;  $P = 0.036$  vs. U-CON; and  $P = 0.005$  vs. T-SCT). Protein carbonyl levels at  $T_{1h}$ ,  $T_{2h}$ , and  $T_{24h}$  were not significantly different from baseline values regardless of the group (Table 2).

**Nitrotyrosine.** Nitrotyrosine levels were not different at baseline among the four groups (NS). An overall training effect (ANOVA trained vs. untrained independently to time point) was observed with higher nitrotyrosine concentrations in untrained subjects compared with their trained counterparts ( $P = 0.01$ ). U-SCT was the only group with a significant increase in nitrotyrosine at  $T_{ex}$  compared with base ( $P = 0.001$ ). Thus U-SCT had higher nitrotyrosine concentrations at  $T_{ex}$  than the other three groups ( $P = 0.048$  vs. U-CON;  $P < 0.0001$  vs. T-CON; and  $P = 0.003$  vs. T-SCT). At  $T_{1h}$ , U-CON, U-SCT, and T-CON exhibited higher nitrotyrosine concentrations than at baseline (Table 2).

#### Antioxidant Markers at Baseline and After Maximal Exercise

**SOD.** No significant inter-group difference was observed in baseline SOD activity. Compared with baseline, a significant increase of SOD activity was observed at  $T_{ex}$  ( $P < 0.0001$ ) and  $T_{1h}$  ( $P = 0.002$ ) in trained subjects, whereas no significant variations were observed in their untrained counterparts (Table 3). Furthermore, SOD activity was significantly higher in the trained subjects than in the untrained ones at  $T_{ex}$  ( $P = 0.015$ ; Fig. 2).

**Catalase.** A training effect was observed at baseline with significantly higher catalase activities in trained subjects than their untrained counterparts ( $P < 0.001$ ). Trained subjects expressed lower activities of catalase at  $T_{ex}$  ( $P < 0.001$ ),  $T_{2h}$  ( $P < 0.001$ ) and  $T_{24h}$  ( $P < 0.001$ ) than at baseline (Table 3).

**GPX.** No significant inter-group differences were observed at baseline for GPX activity. Regardless of the training or

Table 1. Anthropometric, hematologic, and training measurements of the study population

|                    | Untrained                 |                           | Trained                |                        |
|--------------------|---------------------------|---------------------------|------------------------|------------------------|
|                    | CON                       | SCT                       | CON                    | SCT                    |
| %HbS               | N/A                       | $36.2 \pm 3.1$            | N/A                    | $33.2 \pm 3.2$         |
| $\alpha$ -Thal     | N/A                       | 3/8                       | N/A                    | 7/10                   |
| Age, yr            | $22.7 \pm 1.8$            | $23.5 \pm 3.0$            | $24.6 \pm 1.4$         | $23.10 \pm 2.3$        |
| Height, cm         | $169.2 \pm 3.8^*$         | $178.0 \pm 4.4$           | $173.7 \pm 5.5^*$      | $172.1 \pm 3.1^*$      |
| Weight, kg         | $60.1 \pm 5.7$            | $69.5 \pm 5.5^\dagger$    | $69.2 \pm 4.8^\dagger$ | $65.6 \pm 6.1^\dagger$ |
| RBC                | $5.2 \pm 0.7$             | $5.3 \pm 0.5$             | $5.1 \pm 0.1$          | $5.5 \pm 0.2$          |
| Platelets          | $164.1 \pm 81.0^\ddagger$ | $172.7 \pm 82.5^\ddagger$ | $206.6 \pm 95.2$       | $266.9 \pm 54.7$       |
| MAP, W             | $154.1 \pm 16.7^\ddagger$ | $190.4 \pm 11.5^\ddagger$ | $214.6 \pm 11.3$       | $212.5 \pm 10.4$       |
| Max HR, beats/min  | $185 \pm 5$               | $183 \pm 4$               | $174 \pm 3$            | $189 \pm 4$            |
| Relative MAP, W/kg | $2.81 \pm 0.36^\ddagger$  | $2.73 \pm 0.35^\ddagger$  | $3.11 \pm 0.53$        | $3.24 \pm 0.43$        |

Values are presented as means  $\pm$  SD. CON, healthy subjects; SCT, carriers of sickle cell trait; MAP, maximal aerobic power; %HbS, percent of S hemoglobin. \*Significant difference compared with untrained (U)-SCT ( $P < 0.05$ );  $^\dagger$ significant difference compared to U-CON ( $P < 0.05$ );  $^\ddagger$ significant difference compared to trained subjects (CON and SCT,  $P < 0.05$ ).

Table 2. Plasma AOPP, MDA, nitrotyrosine, and protein carbonyl at Base, immediately after a maximal  $T_{ex}$ , and during  $T_{1h}$ ,  $T_{2h}$ ,  $T_{24h}$  in U-CON, U-SCT, T-CON, and T-SCT

|                             | Untrained                       |                                 | Trained                       |                               | Time Effect |
|-----------------------------|---------------------------------|---------------------------------|-------------------------------|-------------------------------|-------------|
|                             | CON                             | SCT                             | CON                           | SCT                           |             |
| AOPP, $\mu$ M               |                                 |                                 |                               |                               |             |
| Base                        | 61.2 $\pm$ 24.7                 | 78.7 $\pm$ 53.6                 | 80.9 $\pm$ 48.5               | 64.4 $\pm$ 16.5               |             |
| $T_{ex}$                    | 127.7 $\pm$ 49.6                | 214.0 $\pm$ 136.9               | 110.4 $\pm$ 66.9              | 123.7 $\pm$ 65.4              | a           |
| $T_{1h}$                    | 73.4 $\pm$ 59.3                 | 125.8 $\pm$ 81.6                | 76.9 $\pm$ 54.5               | 57.4 $\pm$ 49.4               |             |
| $T_{2h}$                    | 99.5 $\pm$ 87.6                 | 136.5 $\pm$ 87.3                | 69.9 $\pm$ 54.1               | 88.3 $\pm$ 64.1               |             |
| $T_{24h}$                   | 86.0 $\pm$ 30.7                 | 124.2 $\pm$ 96.6                | 131.3 $\pm$ 75.5              | 125.6 $\pm$ 69.2              | a           |
| Hb $\times$ training effect |                                 |                                 | e                             |                               |             |
| MDA, $\mu$ M                |                                 |                                 |                               |                               |             |
| Base                        | 23.4 $\pm$ 2.0                  | 20.5 $\pm$ 6.2                  | 27.4 $\pm$ 6.2 <sup>f</sup>   | 27.3 $\pm$ 6.5 <sup>f</sup>   |             |
| $T_{ex}$                    | 24.1 $\pm$ 5.5                  | 32.1 $\pm$ 9.3 <sup>a</sup>     | 27.2 $\pm$ 6.6                | 31.3 $\pm$ 5.2                |             |
| $T_{1h}$                    | 25.2 $\pm$ 4.4                  | 29.4 $\pm$ 5.1 <sup>a</sup>     | 19.8 $\pm$ 5.6 <sup>a,c</sup> | 23.7 $\pm$ 5.2 <sup>a,c</sup> |             |
| $T_{2h}$                    | 25.4 $\pm$ 5.4                  | 28.1 $\pm$ 6.5 <sup>a</sup>     | 23.0 $\pm$ 4.1 <sup>a,c</sup> | 22.7 $\pm$ 4.3 <sup>a,c</sup> |             |
| $T_{24h}$                   | 22.7 $\pm$ 2.0                  | 26.4 $\pm$ 5.2                  | 23.2 $\pm$ 3.8 <sup>a,c</sup> | 21.3 $\pm$ 5.9 <sup>a,c</sup> |             |
| Protein carbonyl, $\mu$ M   |                                 |                                 |                               |                               |             |
| Base                        | 50.5 $\pm$ 12.0                 | 49.2 $\pm$ 11.4                 | 46.8 $\pm$ 14.4               | 51.8 $\pm$ 10.3               |             |
| $T_{ex}$                    | 89.5 $\pm$ 12.0 <sup>f</sup>    | 111.4 $\pm$ 36.6                | 81.6 $\pm$ 16.0 <sup>f</sup>  | 84.8 $\pm$ 25.0 <sup>f</sup>  | a           |
| $T_{1h}$                    | 40.8 $\pm$ 21.9                 | 53.6 $\pm$ 21.3                 | 62.6 $\pm$ 19.3               | 52.8 $\pm$ 15.8               |             |
| $T_{2h}$                    | 36.3 $\pm$ 6.9                  | 43.5 $\pm$ 9.6                  | 52.5 $\pm$ 15.6               | 39.8 $\pm$ 11.6               |             |
| $T_{24h}$                   | 37.1 $\pm$ 7.4                  | 46.7 $\pm$ 18.4                 | 46.2 $\pm$ 13.8               | 41.8 $\pm$ 10.3               |             |
| Nitrotyrosine, nM           |                                 |                                 |                               |                               |             |
| Base                        | 82.8 $\pm$ 28.7                 | 67.6 $\pm$ 20.8                 | 65.4 $\pm$ 21.3               | 95.2 $\pm$ 29.4               |             |
| $T_{ex}$                    | 94.3 $\pm$ 38.2                 | 132.7 $\pm$ 27.7 <sup>a,c</sup> | 97.1 $\pm$ 39.1               | 75.1 $\pm$ 51.2               |             |
| $T_{1h}$                    | 130.5 $\pm$ 22.3 <sup>a,c</sup> | 115.6 $\pm$ 32.9 <sup>a</sup>   | 104.9 $\pm$ 24.4 <sup>a</sup> | 92.0 $\pm$ 27.0               |             |
| $T_{2h}$                    | 102.1 $\pm$ 27.5                | 95.6 $\pm$ 43.4                 | 97.3 $\pm$ 34.3               | 80.0 $\pm$ 30.1               |             |
| $T_{24h}$                   | 76.7 $\pm$ 25.1                 | 83.4 $\pm$ 37.0                 | 47.0 $\pm$ 29.7               | 52.0 $\pm$ 29.8               | c,d         |
| Training effect             |                                 |                                 | g                             | g                             |             |

Values are presented as means  $\pm$  SD. AOPP, advanced oxidation protein products; MDA, malondialdehyde. <sup>a</sup>Significant difference compared with BASE; <sup>b</sup>significant difference compared with the other times; <sup>c</sup>significant difference compared with  $T_{ex}$ ; <sup>d</sup>significant difference compared with  $T_{1h}$  and  $T_{2h}$ ; <sup>e</sup>significant difference compared with the 3 other groups; <sup>f</sup>significant difference compared with U-SCT ( $P < 0.05$ ); <sup>g</sup>significant difference compared with untrained subjects ( $P < 0.05$ ).

hemoglobin status, GPX was significantly higher at  $T_{ex}$ ,  $T_{1h}$ , and  $T_{2h}$  compared with baseline (baseline:  $46.3 \pm 18.8$  vs.  $T_{ex}$ :  $111.6 \pm 31.7$ ,  $P < 0.0001$ , vs.  $T_{1h}$ :  $102.9 \pm 21.6$ ,  $P < 0.0001$ , and vs.  $T_{2h}$ :  $77.9 \pm 21.6$ ,  $P < 0.0001$ ; 4 groups pooled). At  $T_{1h}$  GPX was significantly higher in the trained subjects than in

their untrained counterparts ( $112.0 \pm 14.7$  vs.  $87.7 \pm 9.5$ , respectively,  $P = 0.041$ ; Table 3).

NOx. No significant inter-group difference was observed in baseline NOx. NOx was significantly higher in trained subjects, whatever their hemoglobin status (i.e., T-SCT or T-CON)

Fig. 1. Effect of a maximal exercise test on the plasma malondialdehyde (MDA) in trained sickle cell trait carriers and controls (T-SCT and T-CON) and untrained (U-SCT and U-CON) subjects compared with baseline (Base) at the end of exercise ( $T_{ex}$ ), and during recovery ( $T_{1h}$ ,  $T_{2h}$ ,  $T_{24h}$ ). Values are presented as means  $\pm$  SD. §Significant difference from other 3 groups ( $P < 0.05$ ).

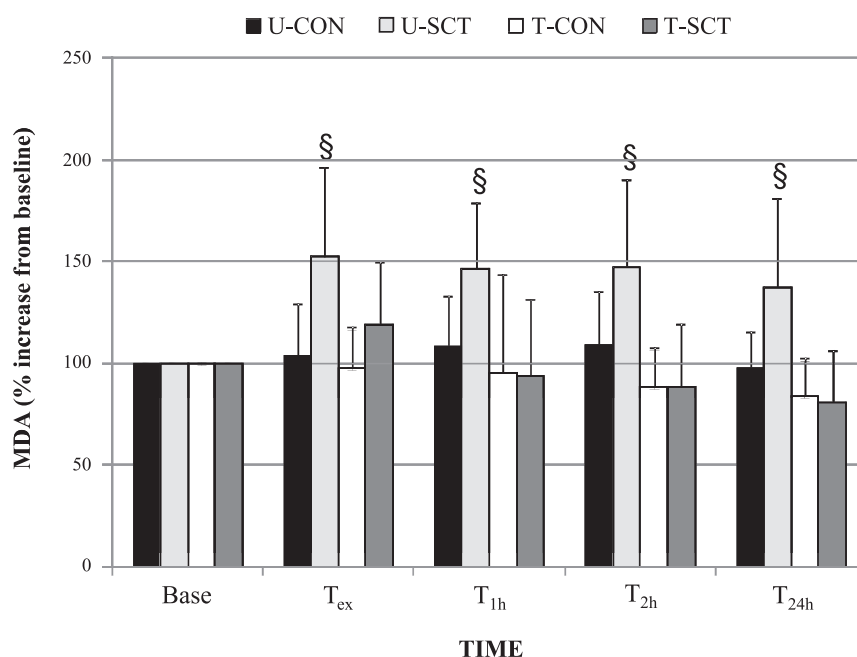


Table 3. Plasma anti-oxidant enzyme activities and NOx at Base, immediately after a maximal exercise test ( $T_{ex}$ ), and during recovery ( $T_{1h}$ ,  $T_{2h}$ ,  $T_{24h}$ ) in U-CON, U-SCT, T-CON, and T-SCT

|                                                                  | Untrained     |               | Trained      |               | Time × Training Effect |
|------------------------------------------------------------------|---------------|---------------|--------------|---------------|------------------------|
|                                                                  | CON           | SCT           | CON          | SCT           |                        |
| SOD, $\mu\text{mol}\cdot\text{ml}^{-1}\cdot\text{min}^{-1}$      |               |               |              |               |                        |
| Base                                                             | 5.6 ± 2.0     | 5.0 ± 1.2     | 3.5 ± 2.3    | 3.4 ± 1.4     |                        |
| $T_{ex}$                                                         | 6.0 ± 1.3     | 4.3 ± 2.5     | 6.5 ± 2.7*   | 8.7 ± 7.7*    | †                      |
| $T_{1h}$                                                         | 5.7 ± 1.4     | 7.8 ± 1.2     | 5.9 ± 1.9*   | 6.3 ± 1.4*    |                        |
| $T_{2h}$                                                         | 5.8 ± 3.4     | 5.3 ± 1.9     | 4.8 ± 1.9    | 4.2 ± 1.4     |                        |
| $T_{24h}$                                                        | 5.1 ± 1.5     | 5.0 ± 1.8     | 5.0 ± 2.0    | 4.0 ± 1.7     |                        |
| Catalase, $\mu\text{mol}\cdot\text{ml}^{-1}\cdot\text{min}^{-1}$ |               |               |              |               |                        |
| Base                                                             | 3.8 ± 1.0     | 4.0 ± 1.8     | 5.0 ± 0.9    | 6.1 ± 1.6     | †                      |
| $T_{ex}$                                                         | 3.4 ± 0.8     | 4.3 ± 1.5     | 3.5 ± 1.6*   | 4.4 ± 1.2*    |                        |
| $T_{1h}$                                                         | 4.6 ± 0.8     | 4.3 ± 1.2     | 4.6 ± 1.7    | 5.1 ± 1.1     |                        |
| $T_{2h}$                                                         | 4.1 ± 1.1     | 3.7 ± 1.1     | 3.9 ± 1.0*   | 3.7 ± 1.2*    |                        |
| $T_{24h}$                                                        | 3.6 ± 0.6     | 3.5 ± 0.6     | 3.5 ± 0.8*   | 3.8 ± 0.5*    |                        |
| GPX, $\mu\text{mol}\cdot\text{ml}^{-1}\cdot\text{min}^{-1}$      |               |               |              |               |                        |
| Base                                                             | 28.1 ± 2.6    | 37.8 ± 19.8   | 54.9 ± 25.6  | 54.0 ± 20.7   |                        |
| $T_{ex}$                                                         | 117.5 ± 31.6* | 100.0 ± 36.2* | 126.5 ± 8.4* | 104.4 ± 38.2* | †                      |
| $T_{1h}$                                                         | 92.3 ± 7.9*   | 83.1 ± 16.1*  | 122.9 ± 9.2* | 104.8 ± 19.7* |                        |
| $T_{2h}$                                                         | 82.0 ± 22.1*  | 68.5 ± 19.0*  | 88.8 ± 26.0* | 73.2 ± 17.2*  |                        |
| $T_{24h}$                                                        | 54.9 ± 32.5   | 60.3 ± 11.3   | 29.3 ± 2.9   | 54.9 ± 24.0   |                        |
| NOx, $\mu\text{mol}\cdot\text{l}^{-1}$                           |               |               |              |               |                        |
| Base                                                             | 20.5 ± 2.9    | 19.7 ± 6.9    | 22.0 ± 5.9   | 19.8 ± 3.1    |                        |
| $T_{ex}$                                                         | 20.6 ± 3.6    | 14.6 ± 7.0    | 26.6 ± 8.0*  | 28.8 ± 11.4*  | †                      |
| $T_{1h}$                                                         | 20.9 ± 6.2    | 22.8 ± 18.0   | 18.5 ± 9.1   | 21.5 ± 9.3    |                        |
| $T_{2h}$                                                         | 24.0 ± 10.7   | 36.1 ± 14.5*‡ | 21.2 ± 7.7   | 19.7 ± 7.6    |                        |
| $T_{24h}$                                                        | 20.8 ± 7.6    | 20.5 ± 10.0   | 21.5 ± 9.1   | 19.9 ± 6.7    |                        |

Values are presented as means ± SD. SOD, superoxide dismutase; NOx, nitric oxide metabolites; GPX, glutathione peroxidase. \*Significant difference compared with BASE ( $P < 0.05$ ); †significant difference compared with untrained ( $P < 0.05$ ); ‡significant difference compared to the three other groups ( $P < 0.05$ ).

at  $T_{ex}$  compared with baseline ( $P = 0.037$ ), whereas NOx remained unchanged in untrained subjects. Consequently, trained subjects had significantly higher NOx values than untrained subjects at  $T_{ex}$  ( $P = 0.001$ ). Moreover, NOx in U-SCT significantly increased at  $T_{2h}$  compared with baseline ( $P = 0.0005$ ) and was significantly higher than the three other groups ( $P = 0.017$  vs. U-CON;  $P = 0.002$  vs. T-CON, and  $P = 0.006$  vs. T-SCT).

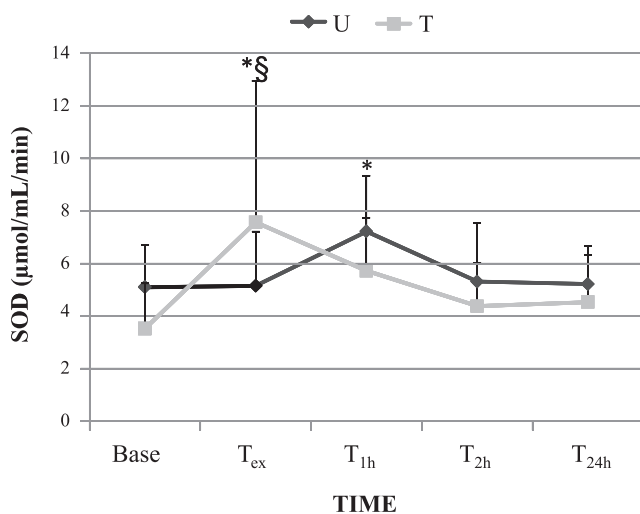


Fig. 2. Effects of a maximal exercise test on the plasma activity of superoxide dismutase (SOD) in untrained (U) or trained (T) subjects at Base, at  $T_{ex}$ , and  $T_{1h}$ ,  $T_{2h}$ , and  $T_{24h}$ . Values represented as means ± SD. \*Significant difference between exercise and Base ( $P < 0.003$ ); §Significant difference between U and T at  $T_{ex}$  ( $P = 0.016$ ).

### Adhesion Molecules

To better understand the correlations between oxidative stress and adhesion molecules, the adhesion data previously presented by Aufradet et al. (3) are summarized in this paragraph. Briefly, basal plasma concentrations of sP-selectin and sE-selectin were not statistically different among the four groups. Plasma sE-selectin significantly increased in all groups at the end of the exercise compared with baseline levels and returned to baseline value 1 h after the end of exercise ( $T_{1h}$ ). Although incremental exercise did not statistically modulate sP-selectin concentrations in T subjects, a significant increase in these concentrations was measured in their untrained counterparts between Base and  $T_{ex}$ . These concentrations returned to basal values 1 h after the end of exercise.

### Correlations

Significant correlations were observed between markers of oxidative stress, nitric oxide, and markers of adhesion. The correlations on the pooled subjects are presented in Table 4. We also found significant relationships between changes (Base vs.  $T_{ex}$ ,  $T_{1h}$ , or  $T_{2h}$ ) in oxidative stress and sE- and sP-selectins or NO metabolism (Table 4). Finally, percentage of HbS was negatively correlated with the NOx increase between baseline and  $T_{ex}$  ( $r = -0.59$ ,  $P = 0.021$ ), i.e., the more the HbS content, the lower the increase in NOx in response to exercise.

### DISCUSSION

The aim of this study was to investigate the impact of regular physical activity on plasma markers of oxidative stress in SCT

Table 4. *Correlations between oxidative stress and adhesion markers (pooled subjects)*

| Variables          | Timepoint                    | Pearson's Correlation | P Value |
|--------------------|------------------------------|-----------------------|---------|
| NOx with           |                              |                       |         |
| AOPP               | Baseline                     | −0.6988               | 0.000   |
| GPX                | Baseline vs. T <sub>1h</sub> | 0.4093                | 0.031   |
| MDA                | Baseline vs. Ex              | −0.3968               | 0.036   |
| AOPP               | Baseline vs. T <sub>1h</sub> | −0.4762               | 0.007   |
| Nitrotyrosine with |                              |                       |         |
| Catalase           | Exercise                     | −0.5694               | 0.002   |
| Carbonyl           | Baseline vs. Ex              | 0.5787                | 0.002   |
| AOPP               | Baseline vs. T <sub>2h</sub> | 0.5671                | 0.004   |
| E-selectin with    |                              |                       |         |
| Catalase           | Exercise                     | −0.3977               | 0.020   |
| MDA                | Baseline vs. T <sub>1h</sub> | 0.3615                | 0.050   |
| P-selectin with    |                              |                       |         |
| AOPP               | Exercise                     | 0.4274                | 0.008   |
| Carbonyl           | T <sub>2h</sub>              | 0.3459                | 0.039   |
| AOPP               | Baseline vs. Ex              | 0.3591                | 0.029   |

Values are presented as Pearson's correlation coefficients and corresponding *P* values.

carriers. In support of our hypotheses, the results of the present study demonstrated that regular physical activity 1) blunted the increase in oxidative stress and the decrease in NO metabolism observed in SCT and 2) upregulated the antioxidant enzymes activities (SOD and GPX) in response to exercise in SCT. In addition, we reported a strong association between changes in various oxidative stress markers in response to exercise and the corresponding changes in sP- and sE-selectins. Although no causality can be assumed, these correlations strengthen the hypothesis that ROS may be involved in endothelial adhesion in HbS carriers.

#### *General Considerations on the Effects of Hemoglobin and Training Status on Hematologic and Training Measurements*

Maximal aerobic power was similar between SCT and control subjects, as supported by previous studies. Conversely, MAP was higher in the trained subjects compared with the untrained subjects. In agreement with several authors (15, 64), we found that trained subjects had a higher platelet count than untrained subjects. The T-SCT subjects had nonsignificantly higher platelet counts than the other three groups. Although an increased platelet count is a risk factor for vaso-occlusive crises in SCD (62), this increase was not significant and is probably due to a training effect rather than a hemoglobin effect.

#### *General Considerations on the Effects of Maximal Exercise and Training on Oxidative Stress and Antioxidant Markers in Healthy Subjects*

Regarding the results obtained in CON, the present study is in complete agreement with data reported in the literature. First, the overall increase in various markers of oxidative stress observed in response to the maximal exercise test is consistent with the results of previous studies (7, 41). Second, strong evidence indicates that exercise training can have a beneficial effect on oxidative stress (21, 28, 41). As supported by Miyazaki et al. (46), trained subjects have an improved ability to endure the stress of a maximal exercise bout. A lower level of oxidative stress was also evident in the trained subjects of our

study. In fact, T-CON subjects had a reduced increase in all markers of oxidative stress in response to the maximal exercise test compared with their untrained counterparts.

It has been reliably shown that individuals partaking in exercise training have higher levels of antioxidant enzymes and exhibit greater protection against exercise-induced oxidative stress (27, 61). The reactive oxygen species produced during exercise training can result in a stimulation of antioxidant defense (39, 57). The exercise-trained subjects in our study produced a significant increase in SOD and GPX, which was accompanied by lower oxidative stress. This decrease in oxidative stress likely occurs due to the improved antioxidant adaptation. Our results are in agreement with several other studies that found an improved antioxidant response relating to exercise training (25, 53).

Our study also shows higher baseline levels of MDA in our trained subjects (16, 21). The high level of resting MDA in trained subjects could be related to the ability of lipid peroxidation products to regulate and modulate cell signaling and gene expression (49). Oxidized lipids can interact with receptors that are known to activate antioxidant enzymes such as catalase and SOD (22) and can improve cellular tolerance against pro-oxidant attacks (49). It is therefore possible that higher levels of lipid peroxidation products, such as MDA, are needed to maintain antioxidant status, such as we saw for catalase and GPX (even if nonsignificant).

#### *Exercise Training Blunts Oxidative Stress in SCT Carriers*

HbS, which represents ~80% of total hemoglobin in SCD patients, can generate a twofold greater quantity of ROS than HbA (2, 23). In SCT carriers, in whom HbS represents ~40% of total hemoglobin, we found that oxidative stress varied little at baseline from healthy subjects. This is in agreement with other studies that found no difference between SCT carriers and healthy subjects at baseline (3, 12, 67). However, in response to acute exercise, SCT carriers can experience higher oxidative stress (12, 36). Our study reinforces this idea as it shows that U-SCT had a significantly greater increase in MDA and AOPP in response to a maximal exercise bout than the healthy subjects. The increased morbidity or mortality in SCT could be attributed to higher levels of oxidative stress (32).

However, the main finding of the present study is that the well-known benefits of exercise training in healthy subjects are well translated in the SCT subjects. In response to the maximal exercise test, T-SCT subjects 1) exhibited lower AOPP, MDA, nitrotyrosine, and protein carbonyl levels than U-SCT, and 2) responded similarly to the T-CON subjects with regard to the protein carbonyl, MDA, and AOPP. In addition to the fact that these results mimic those already seen in many other disease, such as cardiovascular disease (17), diabetes (56), and menopause (53), they emphasize the beneficial effects of exercise training on oxidative stress in SCT carriers.

#### *Antioxidant Defense System is Improved in Exercise-Trained SCT Carriers*

The improvement in the antioxidants defense system seen in exercise training is due to a ROS-generated stimulation triggering antioxidant enzyme activation (39, 57). The maximal exercise test that normally increases oxidative stress was met by a concomitant increase in antioxidant enzyme defenses, in



all but the U-SCT carriers. Repeated habitual exercise can cause antioxidants to increase in response to the repeated oxidative stress. This has been supported in vitro where the treatment of pro-oxidants stimulated a significant increase in antioxidants (18). Interestingly, this did not occur in U-SCT subjects, as they had a delayed response of SOD and a reversed response of catalase. This supports studies that showed levels of SOD and catalase decrease in proportion to disease severity (13, 59). The response to SOD and catalase could indicate an impaired antioxidant status, meaning a reduced ability to buffer the excess oxidative stress.

The trait by itself can result in an increase in oxidative stress and impairment in NO, and when paired with an acute stress such as a maximal exercise test, this response is augmented, as observed with U-SCT. However, exercise training is able to stimulate antioxidants to respond to the overload of oxidative stress. This improved response to oxidative stress allows SCT carriers to respond similarly to CON subjects, as demonstrated by our study. This indicates that, as far as oxidative stress is concerned, training can override the negative consequence of SCT, rendering these subjects as controls.

### Nitric Oxide

Nitric oxide, an important mediator of vasodilation, has been shown to be amplified in response to exercise training (21, 40, 41, 44). The present study supports these findings, as the SCT and CON trained subjects significantly increased NOx immediately after the exercise test, whereas the untrained subjects slightly decreased. Our study suggests that exercise training may inhibit NO degradation through an upregulation of antioxidants and a decrease in ROS. We found that the percent change (% $\Delta$ ) of NOx levels were positively correlated with GPX levels at  $T_{1h}$  and were negatively correlated with MDA levels (% $\Delta$ ) at  $T_{ex}$  and AOPP levels (% $\Delta$ ) at  $T_{1h}$ . Nitrotyrosine, which represents the nitration activity of peroxynitrite (ONOO<sup>-</sup>) produced through the reaction of NO and superoxide anion (O<sub>2</sub><sup>-</sup>), tended to increase in the untrained subjects compared with the trained subjects ( $P = 0.055$ ) and was significantly higher in the U-SCT carriers compared with T-SCT carriers ( $P = 0.003$ ). This is in accordance with other studies that found a decrease in nitrotyrosine levels in exercise-trained subjects (37, 53). Although our correlations do not suggest causality, these results 1) could indicate a reduced O<sub>2</sub><sup>-</sup> production and 2) suggest an increase in NO bioavailability as a result of reduced oxidative stress (21, 53) in trained SCT carriers than in their untrained counterparts.

The improvement in NOx levels in response to physical activity level is reflected in the SCT subjects as well. T-SCT subjects had a similar improvement in NOx response as the T-CON subjects. Interestingly, the U-SCT had a delayed peak in NOx at  $T_{2h}$ , which was associated with an elevation in oxidative stress. These results emphasize the overall impairment in response to an exhaustive exercise bout. An impaired NO bioavailability is associated with an increase in ROS, hemolysis (58), eNOS uncoupling due to hemolysis-induced arginase, and cell adhesion (35). Because NO has potent anti-adhesive properties which downregulate adhesion molecule expression maintaining proper endothelial cell function and vasodilation, a NO impairment can increase cell adhesion (35). This is supported by Aufradet et al. (3), who found a

significant increase in VCAM-1 levels in untrained SCT subjects immediately after exercise (U-SCT:  $1,738 \pm 98$  ng/ml vs. T-SCT:  $1,248 \pm 131$  ng/ml;  $P < .05$ )—the exact same time point as the decreased NOx response in U-SCT carriers. As ROS markers were positively correlated with sP- and sE-selectins and negatively correlated with antioxidant markers, these data suggest that a NO-induced increase in oxidative stress could exacerbate endothelial and/or platelets activation. However, it should be noted that there were no differences between sP-selectin and sE-selectin in the different subjects. Therefore, the training-induced decrease in oxidative stress and increase in NO bioavailability could be favorable for the health of SCT carriers involved in regular exercise by dampening risk of sickling, morbidity, and mortality.

This study suggests that exercise training can improve the response to oxidative stress in SCT carriers. However, at this time, most studies involving different intensities and durations of exercise have found conflicting results in relation to coagulation activity, RBC deformability, inflammation, and adhesion in SCT [see review by Connes et al. (11)]. However, these events may be compounded by other factors such as heat stress, dehydration, and poor physical conditioning. Baskurt and Meiselman (4), suggested that exercise utilizes the vascular autoregulatory reserve to maintain homeostasis, yet even minor vascular and hemorheologic perturbations in SCT carriers may be augmented in response to exercise.

### Conclusion

In conclusion, we found that although there is relatively little difference between SCT carriers and healthy subjects at rest, a maximal exercise test can inundate the oxidative stress response in U-SCT subjects. We also found that training can reduce the oxidative stress in response to exercise of SCT carriers. Training improves antioxidant and NO availability that can thereafter regulate ROS production. These effects could result in decreased endothelial activation. This study does not allow us to make definitive conclusions about the direct causality between exercise training and oxidative stress and NO improvements. Further information could be concluded using longitudinal studies which focus on mechanistic pathways and more directly evaluate endothelial dysfunction using methods such as flow mediated dilation.

Finally, we believe that the beneficial effects seen in SCT carriers in this study could translate well in SCD patients as well. Although there are large clinical differences between SCT and SCD, both are overwhelmed by an increase in oxidative stress. Because we have shown that exercise training can decrease oxidative stress and improve antioxidant and nitric oxide responses, we believe that the complications known in SCD, such as vaso-occlusion crisis, that are linked to oxidative stress (26, 38) could potentially be reduced. Therefore, an adapted exercise training program could be a relevant option to control the cardiovascular complications of this pathology.

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## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

## AUTHOR CONTRIBUTIONS

Author contributions: E.N.C., C.M., C.F., L.F., E.A., H.D., A.F., L.A.M., and V.P. performed experiments; E.N.C., C.M., and V.P. analyzed data; E.N.C. interpreted results of experiments; E.N.C. prepared figures; E.N.C., C.M., and L.A.M. drafted manuscript; E.N.C., C.M., C.F., L.F., H.D., E.C.-S., P.T., L.A.M., and V.P. edited and revised manuscript; S.O.-E., L.A.M., and V.P. conception and design of research; L.A.M. and V.P. approved final version of manuscript.

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**IX.1c Editorial: Research in Athletes with Sickle Cell Trait: Just Do It**



**Title:**

Research in Athletes with Sickle Cell Trait: Just Do It

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**Running Head:**

Athletes with Sickle Cell Trait

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## Research in Athletes with Sickle Cell Trait: Just Do It

The sickle cell trait (SCT) genotype, a condition found in an estimated 300 million people worldwide and 8-9% of African Americans, is characterized by the heterozygous inheritance of a single defective beta globin gene. Inherited in the homozygous form, the beta globin gene mutation leads to sickle cell disease (SCD). Patients with SCD have profound clinical manifestations that can be associated with significant morbidity and mortality. On the contrary, individuals with SCT do not have a disease.

The sickle cell trait phenotype is almost always benign. Rarely, under ill-defined conditions of environmental and physical extremes, individuals with sickle cell trait can have clinical symptoms. Despite their exceedingly uncommon occurrence, several complications can occur, the most newsworthy of which is a presumptive association between SCT and sudden death during intense physical activity. The causative pathophysiologic mechanisms and risk factors that lead to the development of symptoms in individuals with SCT remain unknown.

In 2010, the National Collegiate Athletic Association (NCAA) implemented a mandate requiring all incoming Division I student athletes to undergo testing for SCT[1]. Should they choose not to be tested, an athlete has the option of either showing proof of a prior test or signing a release of liability waiver absolving their academic institution and the NCAA from liability. Since its inception, this policy has been mired in controversy. The polemic nature of the subject stems from the fact that the mandate, a policy issued in response to litigation rather than one driven by evidence-based science, has impassioned implications pertaining to genetic privacy, genetic discrimination, social stigmatization, and racial profiling. The mandate, in our opinion, comes precariously close to violating the fundamental ethical principles of beneficence and non-maleficence. Numerous leading organizations including the American Society of Hematology, the Department of Health and Human Service's Secretary's Advisory Committee on Heritable Disorders in Newborns and Children, and the Sickle Cell Disease Association of America, have issued policy statements in opposition to the NCAA's mandated screening[2, 3]. Moreover, these organizations strongly support and advocate for organized biomedical and epidemiologic research investigating the rare but potential pathophysiologic implications of sickle cell trait.

In their study titled, Exercise Training Blunts Oxidative Stress in Sickle Cell Trait Carriers, Chirico et al, (*reference to pages in this issue*) investigated the impact of regular physical activity on plasma markers of oxidative stress, nitric oxide metabolism, and endothelial adhesion in individuals with sickle cell trait compared to controls. In this paper, the authors present data to show that exercise has a potential modulatory effect on oxidative stress and nitric oxide metabolism in individuals with sickle cell trait. The authors compared exercise trained SCT carriers to their untrained counterparts and found that physical activity could be a conceivably viable method of controlling oxidative stress. Although there are large clinical differences between SCT and SCD, the authors suggest that exercise training could also have potentially beneficial effects on patients with SCD. This paper clearly shows that carefully conducted studies need to be performed in order to fully define and characterize modifiable risk factors in carriers of the sickle beta globin gene mutation.

Unfortunately, very few well-controlled studies have been performed correlating

exercise physiology to the genetic, biologic and clinical aspects of individuals with SCT. The controversy over the NCAA mandate has generated a national discussion, the theme of which underscores and substantiates the need for focused research that better defines risk factors for these individuals. Such studies are in our view essential. Promising young high school athletes who pursue their education and passion for sport would be far better served by well-conducted studies that define and characterize risk factors, thereby enabling us to provide evidence-based anticipatory guidance, than by the uncertainty and possible stigma generated by the currently mandated genetic test. Chirico et al show that such studies can be done. We charge others to simply *just do it* as well.

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**IX.1d Effect of  $\alpha$ -thalassemia on exercise-induced oxidative stress in sickle cell trait**

## Effect of $\alpha$ -thalassaemia on exercise-induced oxidative stress in sickle cell trait

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### Abstract

**Aim:** Alpha-thalassaemia is known to reduce intra-erythrocyte HbS (sickle haemoglobin) concentration in sickle cell trait (SCT) subjects. Because HbS was shown to increase oxidative stress, the purpose of this study was to assess the effects of the coexistence of  $\alpha$ -thalassaemia and SCT on oxidative stress markers and nitric oxide (NO) metabolism after an acute physical exercise.

**Methods:** Forty subjects (age:  $23.5 \pm 2.21$  years), SCT carriers (HbAS) or healthy subjects (HbAA), with ( $-\alpha T$ ) or without ( $-N\alpha T$ ) an associated  $\alpha$ -thalassaemia took part in the study. Plasma markers of oxidative stress [advanced oxidation protein products (AOPP), protein carbonyl, malondialdehyde (MDA) and nitrotyrosine], anti-oxidant defences and NO metabolism (NOx) were measured at rest ( $T_{rest}$ ), immediately following an incremental maximal exercise test ( $T_{ex}$ ) and during recovery ( $T_{1h}$ ,  $T_{2h}$  and  $T_{24h}$ ).

**Results:** Malondialdehyde expressed as the percentage of changes from baseline was significantly higher in the HbAS- $N\alpha T$  compared with HbAS- $\alpha T$  during recovery ( $+36.3 \pm 14.1\%$  vs.  $-1.8 \pm 13.2\%$  at  $T_{1h}$ ,  $P = 0.02$ ;  $+36.6 \pm 13.4\%$  vs.  $-11.4 \pm 12.5\%$  at  $T_{2h}$ ,  $P = 0.004$  and  $+24.1 \pm 12.3\%$  vs.  $-14.4 \pm 11.5\%$  at  $T_{24h}$ ,  $P = 0.02$  in HbAS- $N\alpha T$  vs. HbAS- $\alpha T$ ). Compared with HbAS- $N\alpha T$ , HbAS- $\alpha T$  had a higher NOx change from baseline at  $T_{ex}$  ( $-23.4 \pm 20.6\%$  vs.  $+57.7 \pm 19.3\%$ , respectively;  $P = 0.005$ ) and lower nitrotyrosine change from baseline at  $T_{1h}$  ( $+7.2 \pm 22.2\%$  vs.  $+93.5 \pm 29.3\%$ , respectively;  $P = 0.04$ ).

**Conclusion:** All these data suggest that the presence of  $\alpha$ -thalassaemia may blunt the higher level of oxidative stress and the impaired bioavailability of NO observed in the SCT carriers.

**Keywords** haemoglobinopathy, lipid peroxidation, nitric oxide metabolism, vaso-occlusion.

Sickle cell disease (SCD) is characterized by a genetic mutation leading to the production of abnormal haemoglobin (HbS). In a low oxygen tension environment, the deoxygenated HbS is subjected to polymerization, inducing the sickling process of the red blood cells (RBCs). This process alters the normal biconcave shape into stiffened and irregular RBCs, notably leading to vascular occlusions (Chiang & Frenette 2005). In addition, the sickling-induced weakening of the RBCs leads to a haemolytic anaemia.

Sickle cell trait (SCT, heterozygous form of the disease) is characterized by the presence of both normal haemoglobin (HbA) and HbS in the erythrocytes (HbAS) and is often considered asymptomatic and benign during physical activity (Connes *et al.* 2008). However, several studies reported complications and cases of sudden death of SCT carriers, under specific circumstances such as exertional exercises (Eichner 2010).

Vaso-occlusive crises leading to localized ischaemia-reperfusion and haemolytic events enhance production of reactive oxygen species (ROS) (Aslan & Freeman 2007). Increasing evidence indicates that ROS play a crucial role in SCD pathophysiology (Wood & Granger 2007). Through the reaction of nitric oxide (NO) with the superoxide anion ( $O_2^{\cdot-}$ ) to form peroxynitrite ( $ONOO^-$ ), ROS impair the bioavailability of NO, subsequently inducing endothelial dysfunction (Thomas *et al.* 2008).

Intense physical exercise results in an elevated energy requirement, increasing to a large extent the oxidative phosphorylation. This increase in  $O_2$  flux in the mitochondria can lead to an overproduction of  $O_2^{\cdot-}$  and oxygen-derived intermediates (Sen 1995) inducing ROS overproduction (Leeuwenburgh & Heinecke 2001) and subsequent oxidative damage in the biological molecule (Davies *et al.* 1982, Pialoux *et al.* 2006). Moreover, intense exercise can also be seen as a stimulus, which can be used to reveal the differences between subjects' susceptibility to oxidative stress (Pialoux *et al.* 2006). Whereas oxidative stress was extensively studied in various disease states, only few studies to our knowledge have tested the level of oxidative stress in SCT carriers. Das *et al.* 1993 reported a trend towards an increased lipid peroxidation in erythrocytes of SCT carriers in response to a maximal graded exercise. Conversely, Tripette *et al.* 2010 found no changes in plasma thiobarbituric acid reacting substance (TBARS), reduced membrane thiols and plasma nitrite levels after moderate exercise in SCT carriers. This latter study suggests that a high intensity exercise might be required to significantly increase oxidative stress in SCT carriers.

Alpha-thalassaemia is an inherited blood disorder caused by the deletion of a part of the  $\alpha$ -globin genes

located on the chromosome 16. Monchanin *et al.* 2005 reported that  $\alpha$ -thalassaemia tends to minimize the severity of haemorheological complications in SCT carriers. Indeed, by decreasing the percentage of HbS in erythrocytes,  $\alpha$ -thalassaemia provides a relative protection against the sickling process (Harkness 1989). Therefore, it can be hypothesized that  $\alpha$ -thalassaemia may reduce oxidative stress induced by acute physical exercise in SCT carriers. However, the oxidative stress and NO metabolism in subjects carrying the dual haemoglobinopathy have never been studied so far.

Thus, the aim of the current study was to assess the consequences of  $\alpha$ -thalassaemia and SCT on oxidative stress. More specifically, we investigated plasma markers of protein and lipid oxidation, protein nitration, antioxidant status and NO metabolism in response to maximal exercise in control,  $\alpha$ -thalassaemic, SCT carriers and subjects with dual haemoglobinopathy.

## Materials and methods

### Ethical approval

All subjects involved in this study gave their written informed consent to participate in the study, which was approved by the local ethics committee and complied with the guidelines set by the Declaration of Helsinki.

### Subjects

Forty male subjects were recruited from students at the University of Yaounde II (Soa, Cameroon). They were selected and divided into four groups according to their haemoglobin profile (HbAA-N $\alpha$ T,  $n = 14$ , HbAA- $\alpha$ T,  $n = 8$ ; HbAS-N $\alpha$ T,  $n = 8$  and HbAS- $\alpha$ T,  $n = 10$ ). Prior to their admission in the protocol, they were submitted to a medical examination, including anthropometric measurements and a blood screening for SCT and  $\alpha$ -thalassaemia diagnosis, measurements of haematological parameters and human immunodeficiency virus (HIV) detection. The subjects were excluded from the study if they had hypertension, HIV, experienced strokes or had a malaria crisis within the past 3 months.

### Study design and exercise test

All the experiments were conducted in the General Hospital of Yaounde (Cameroon). Subjects refrained from taking part in strenuous exercise the day before the exercise test, which consisted in an incremental exercise on a cycle ergometer (818E; Monark, Stockholm, Sweden) up to volitional exhaustion. After a 5-min warm up at 30 W, the initial work load of

70 W was increased stepwise by 35 W, every 3 min until exhaustion. The pedalling frequency was fixed at 70 rpm throughout the test. HR was continuously monitored (Polar Electro, Kempele, Finland) to assess maximal heart rate ( $HR_{max}$ , beats  $min^{-1}$ ). Maximal aerobic power (MAP, W) was determined by linear interpolation from the HR vs. work rate curve. During this test, blood samples were collected in EDTA tubes at rest ( $T_{rest}$ ), immediately after the end of exercise ( $T_{ex}$ ), and at 1, 2 and 24 h ( $T_{1h}$ ,  $T_{2h}$ ,  $T_{24h}$ ) into recovery. Plasma was obtained by centrifugation for 10 min at 4 °C immediately after the blood withdrawal, then separated into aliquots, frozen in liquid nitrogen and stored at -80 °C until analysis. These samples were used to quantify plasma levels of oxidative markers [advanced oxidation protein products (AOPP), malondialdehyde (MDA), protein carbonyl and nitrotyrosine], antioxidant enzymatic activities [superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase] and nitric oxide availability (NOx). Plasma ferric reducing antioxidant power (FRAP) was only analysed at  $T_{rest}$ ,  $T_{ex}$  and  $T_{1h}$ .

#### Sickle cell trait, $\alpha$ -thalassaemia diagnosis and haematological parameters

Testing for the presence of HbS was carried out by citrate agar electrophoresis. The various Hb isolated were quantified by ion-exchange high-performance liquid chromatography (Variant I, Beta Thal Short Program; Bio-Rad Laboratories, Hercules, CA, USA). The results for SCT were determined by the presence of HbS but <50% of total Hb. To diagnose  $\alpha$ -thalassaemia, a single-tube multiplex-PCR technique was used, according to the method described by Chong *et al.* 2000. Among the several subtypes of  $\alpha$ -thalassaemias, the only one form detected in this population was the heterozygous, marked by a depletion of 3.7 kb of DNA, and containing one of the two linked  $\alpha$ -globin genes ( $\alpha/\alpha^{3,7}$ ). The measurements of haematological parameters such as amount of RBC ( $10^{-6}$  cells  $\mu L^{-1}$ ), haemoglobin concentration ([Hb]), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were performed using a haematology analyser (Abbott Cell Dyn 1800 haematology).

#### Biochemical assays

**Plasma advanced oxidation protein products (AOPP).** Plasma advanced oxidation protein products were measured according to the semi-automated methods developed by Witko-Sarsat *et al.* 1996. The plasma concentrations were determined by spectrophotometry and were calibrated with a chloramine-T

solution that absorbs at 340 nm in the presence of potassium iodide. The absorbance of the reaction was read at 340 nm. AOPP concentrations were expressed as  $\mu mol L^{-1}$  of chloramine-T equivalents.

**Protein Carbonyl.** Oxidative damage to proteins was also assessed by the spectrophotometric quantification of carbonyl groups at 380 nm, using 2,4-dinitrophenylhydrazine (DNPH), according to a modified method of Levine *et al.* 2000.

**Malondialdehyde.** Concentrations of MDA, as thio-barbituric reactive substances, were determined as previously described (Pialoux *et al.* 2006). The pink chromogen was extracted with *n*-butanol, and its absorbance was measured at 532 nm by spectrophotometry using 1,1,3,3-tetraethoxypropane as standard. Although MDA assay is often considered as a moderately sensitive and specific technique, it is still widely used as an oxidative stress marker.

**Superoxide dismutase.** The quantitative determination of the SOD activity was performed using the method described by Oberley and Spitz 1984. Superoxide dismutase activity was determined by the degree of inhibition of the reaction between superoxide radicals, produced by a hypoxanthine – xanthine oxidase system, and nitroblue tetrazolium.

**Catalase.** Catalase activity was assessed by the method of Johansson and Borg 1988 who used hydrogen peroxide ( $H_2O_2$ ) as substrate and formaldehyde as standard.

**Glutathione peroxidase.** Glutathione peroxidase was determined by the modified method of Paglia and Valentine 1967. Glutathione peroxidase activity was determined as the rate of oxidation of NADPH to  $NADP^+$  after addition of glutathione reductase (GR), reduced glutathione (GSH) and NADPH, using  $H_2O_2$  as substrate.

**Ferric reducing antioxidant power.** Plasma FRAP was measured by spectrophotometry using the manual method of Benzie and Strain 1996. Ferric reducing antioxidant power concentration was calculated using an aqueous solution of known  $Fe^{2+}$  concentration ( $Fe SO_4 \cdot 7H_2O$ ) as standard. Plasma was mixed with working FRAP solution, warmed at 37 °C, made up for buffer acetate, 2,4,6-Tris(2-pyridyl)-s-triazine, and ferric chloride solution ( $FeCl_3 \cdot 6H_2O$ ).

**Nitrite and nitrate (NOx).** To measure NO production, we measured the sum of nitrite and nitrate concentrations. After nitrate reduction by nitrate reductase,



the fluorimetric quantification of NOx was based upon the reaction of nitrite with 2,3-diaminonaphthalene as previously described (Misko *et al.* 1993).

**Nitrotyrosine.** Concentrations of plasma nitrotyrosine, as end product of protein nitration by ONOO<sup>-</sup>, were measured by ELISA as previously described (Galiñanes & Matata 2002).

### Statistics

Statistical analyses were performed using Statistica (Version 8.0; StatSoft, Tulsa, OK, USA), and data were reported as mean  $\pm$  SD. The changes from baseline have been calculated as the mean of the individual percentage change from baseline. Two-way repeated-measure analysis of variance (ANOVA) followed by Fisher's test were used for multiple comparisons between four groups, at different times. The anthropometric characteristics, haematological parameters and MAP were analysed using a two-way ANOVA procedure followed by post hoc tests.

### Results

#### Anthropometric characteristics and maximal aerobic power measurement

Anthropometric data and MAP values of our cohort are presented in Table 1. Age, weight, HR<sub>max</sub> and MAP values were not different among the four groups studied. HbAS-N $\alpha$ T subjects were significantly taller than their HbAA-N $\alpha$ T counterparts.

#### Haematological parameters

As shown in Table 1, the percentage of HbS and MCV values were significantly lower in HbAS- $\alpha$ T subjects than in HbAS-N $\alpha$ T ( $P < 0.001$ ).  $\alpha$ -thalassaemic subjects (HbAS- $\alpha$ T and HbAA- $\alpha$ T) displayed higher RBC count and lower MCH than their control counterparts. Haematocrit did not differ among the groups.

#### Markers of lipid and protein oxidation in plasma

Resting concentrations of AOPP, MDA and protein carbonyl are presented in Table 2. Resting values of AOPP and protein carbonyl are not different among the groups. Regardless of the group, MDA ( $+14.1 \pm 8.6\%$ , *time effect*:  $P < 0.01$ ) was higher at T<sub>ex</sub> compared with T<sub>rest</sub>. Malondialdehyde concentrations at T<sub>rest</sub> showed higher values in HbAS- $\alpha$ T as compared to HbAS-N $\alpha$ T ( $P < 0.05$ ). However, when analyzing the percentages of MDA change from baseline, HbAS-N $\alpha$ T values were significantly higher than in HbAS- $\alpha$ T during the whole period of recovery ( $+36.3 \pm 14.1\%$  vs.  $-1.8 \pm 13.2\%$  at T<sub>1h</sub>,  $P = 0.02$ ,  $+36.6 \pm 13.4\%$  vs.  $-11.4 \pm 12.5\%$  at T<sub>2h</sub>,  $P = 0.004$  and  $+24.1 \pm 12.3\%$  vs.  $-14.4 \pm 11.5\%$  at T<sub>24h</sub>,  $P = 0.02$ , respectively; Fig. 1b). Similarly to MDA, AOPP significantly increased in T<sub>ex</sub> ( $+98.5 \pm 104.8\%$ ), *time effect* ( $P < 0.01$ ) regardless of the group. In addition, independent of  $\alpha$ -thalassaemia, HbAS-N $\alpha$ T increased significantly AOPP between T<sub>rest</sub> and T<sub>ex</sub> ( $+162.3 \pm 50.1\%$ , *time effect*:  $P < 0.01$ ; Fig. 1a) while HbAA-N $\alpha$ T did not change significantly. Finally,

**Table 1** Anthropometric, fitness characteristics and resting haematology of the study population

| Values                                              | HbAA-N $\alpha$ T ( $n = 14$ ) | HbAA- $\alpha$ T ( $n = 8$ ) | HbAS-N $\alpha$ T ( $n = 8$ ) | HbAS- $\alpha$ T ( $n = 10$ ) |
|-----------------------------------------------------|--------------------------------|------------------------------|-------------------------------|-------------------------------|
| Age (yr)                                            | 23.6 $\pm$ 1.8                 | 23.8 $\pm$ 2.1               | 24.1 $\pm$ 3.0                | 22.6 $\pm$ 2.0                |
| Height (cm)                                         | 170.5 $\pm$ 4.4                | 173.1 $\pm$ 6.2              | 175.8 $\pm$ 4.0*              | 173.9 $\pm$ 5.3               |
| Weight (Kg)                                         | 63.1 $\pm$ 7.0                 | 67.3 $\pm$ 6.5               | 66.9 $\pm$ 6.6                | 67.7 $\pm$ 5.8                |
| MAP (W)                                             | 180 $\pm$ 32                   | 193 $\pm$ 85                 | 190 $\pm$ 41                  | 213 $\pm$ 24                  |
| HR <sub>max</sub> (beats.min <sup>-1</sup> )        | 180 $\pm$ 16                   | 182 $\pm$ 10                 | 187 $\pm$ 12                  | 185 $\pm$ 11                  |
| % HbS                                               | —                              | —                            | 38.09 $\pm$ 0.98              | 31.63 $\pm$ 0.90 <sup>†</sup> |
| RBC (10 <sup>-6</sup> cells $\mu$ L <sup>-1</sup> ) | 4.95 $\pm$ 0.36                | 5.54 $\pm$ 0.66*             | 5.20 $\pm$ 0.53               | 5.62 $\pm$ 0.42*              |
| Haematocrit (%)                                     | 43.3 $\pm$ 2.7                 | 44.5 $\pm$ 5.0               | 43.1 $\pm$ 3.5                | 43.4 $\pm$ 2.4                |
| MCV (fL)                                            | 87.6 $\pm$ 3.7                 | 80.5 $\pm$ 3.7*              | 83.2 $\pm$ 3.2*               | 77.4 $\pm$ 2.7* <sup>†</sup>  |
| MCH (pg)                                            | 28.2 $\pm$ 1.4                 | 25.7 $\pm$ 1.3*              | 27.4 $\pm$ 1.1                | 25.0 $\pm$ 0.9* <sup>†</sup>  |
| MCHC (g/100ml)                                      | 32.1 $\pm$ 0.8 <sup>†</sup>    | 32.0 $\pm$ 0.4 <sup>†</sup>  | 32.9 $\pm$ 0.7                | 32.2 $\pm$ 0.4 <sup>†</sup>   |

Values are presented as means  $\pm$  SD. HbAA-N $\alpha$ T, control healthy subjects (without sickle cell trait and  $\alpha$ -thalassaemia); HbAA- $\alpha$ T,  $\alpha$ -thalassaemic subjects (without sickle cell trait); HbAS-N $\alpha$ T, sickle cell trait carriers (without  $\alpha$ -thalassaemia); HbAS- $\alpha$ T,  $\alpha$ -thalassaemic sickle cell trait carriers; HR<sub>max</sub>, maximal heart rate; MAP, maximal aerobic power; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; RBC, red blood cell.

\*Data are significantly different from HbAA-N $\alpha$ T group at  $P < 0.05$ .

<sup>†</sup>Data are significantly different from HbAS-N $\alpha$ T group at  $P < 0.05$ .



**Table 2** Plasma AOPP, MDA, protein carbonyl, NOx and nitrotyrosine at rest ( $T_{\text{rest}}$ ), immediately after a maximal exercise ( $T_{\text{ex}}$ ) and during the recovery ( $T_{1\text{h}}$ ,  $T_{2\text{h}}$  and  $T_{24\text{h}}$ ) in HbAA-N $\alpha$ T, HbAA- $\alpha$ T, HbAS-N $\alpha$ T and HbAS- $\alpha$ T

| Values                                  | Times             | HbAA-N $\alpha$ T<br>( $n = 14$ ) | HbAA- $\alpha$ T<br>( $n = 8$ ) | HbAS-N $\alpha$ T<br>( $n = 8$ ) | HbAS- $\alpha$ T<br>( $n = 10$ ) | Time<br>effect |
|-----------------------------------------|-------------------|-----------------------------------|---------------------------------|----------------------------------|----------------------------------|----------------|
| AOPP ( $\mu\text{mol L}^{-1}$ )         | $T_{\text{rest}}$ | 75.8 $\pm$ 47.2                   | 68.1 $\pm$ 31.8                 | 58.8 $\pm$ 29.9                  | 81.8 $\pm$ 42.4                  | *              |
|                                         | $T_{\text{ex}}$   | 110.5 $\pm$ 51.0                  | 127.5 $\pm$ 71.4                | 124.6 $\pm$ 47.7                 | 201.9 $\pm$ 139.8                |                |
|                                         | $T_{1\text{h}}$   | 70.8 $\pm$ 55.9                   | 81.5 $\pm$ 56.9                 | 105.0 $\pm$ 80.8                 | 75.6 $\pm$ 67.7                  |                |
|                                         | $T_{2\text{h}}$   | 82.1 $\pm$ 60.5                   | 83.2 $\pm$ 85.2                 | 134.9 $\pm$ 89.6                 | 89.7 $\pm$ 62.6                  |                |
|                                         | $T_{24\text{h}}$  | 92.3 $\pm$ 53.2                   | 138.0 $\pm$ 70.2                | 135.4 $\pm$ 105.4                | 115.8 $\pm$ 55.4                 |                |
| MDA ( $\mu\text{mol L}^{-1}$ )          | $T_{\text{rest}}$ | 25.0 $\pm$ 4.0                    | 25.6 $\pm$ 5.9                  | 21.2 $\pm$ 6.2                   | 27.5 $\pm$ 6.7 <sup>†</sup>      | *              |
|                                         | $T_{\text{ex}}$   | 29.1 $\pm$ 4.8                    | 21.3 $\pm$ 4.1 <sup>§</sup>     | 28.5 $\pm$ 4.1                   | 34.4 $\pm$ 7.8 <sup>†</sup>      |                |
|                                         | $T_{1\text{h}}$   | 23.4 $\pm$ 5.4                    | 21.8 $\pm$ 6.1                  | 27.0 $\pm$ 4.4                   | 25.0 $\pm$ 6.9                   |                |
|                                         | $T_{2\text{h}}$   | 24.0 $\pm$ 3.6                    | 24.7 $\pm$ 6.4                  | 27.3 $\pm$ 6.9                   | 22.8 $\pm$ 4.0                   |                |
|                                         | $T_{24\text{h}}$  | 24.1 $\pm$ 3.1                    | 21.5 $\pm$ 1.7                  | 24.0 $\pm$ 5.6                   | 22.8 $\pm$ 6.6                   |                |
| Protein Carbonyl (nmol.<br>mL $^{-1}$ ) | $T_{\text{rest}}$ | 47.5 $\pm$ 9.4                    | 49.3 $\pm$ 16.1                 | 51.2 $\pm$ 9.6                   | 50.3 $\pm$ 11.6                  | *              |
|                                         | $T_{\text{ex}}$   | 88.7 $\pm$ 8.9                    | 82.3 $\pm$ 17.9                 | 94.5 $\pm$ 46.4                  | 98.7 $\pm$ 24.1                  |                |
|                                         | $T_{1\text{h}}$   | 53.2 $\pm$ 22.5                   | 52.3 $\pm$ 24.8                 | 53.8 $\pm$ 15.6                  | 52.8 $\pm$ 20.0                  |                |
|                                         | $T_{2\text{h}}$   | 47.7 $\pm$ 14.4                   | 43.0 $\pm$ 15.7                 | 43.0 $\pm$ 11.4                  | 40.6 $\pm$ 10.5                  |                |
|                                         | $T_{24\text{h}}$  | 39.3 $\pm$ 5.7                    | 44.4 $\pm$ 15.5                 | 44.7 $\pm$ 14.9                  | 43.7 $\pm$ 14.7                  |                |
| NOx ( $\mu\text{mol L}^{-1}$ )          | $T_{\text{rest}}$ | 20.73 $\pm$ 5.66                  | 21.99 $\pm$ 3.95                | 21.19 $\pm$ 6.21                 | 18.48 $\pm$ 3.70                 |                |
|                                         | $T_{\text{ex}}$   | 21.01 $\pm$ 3.36                  | 27.01 $\pm$ 8.55                | 15.65 $\pm$ 8.44                 | 27.87 $\pm$ 11.69                |                |
|                                         | $T_{1\text{h}}$   | 20.42 $\pm$ 10.67                 | 18.63 $\pm$ 4.05                | 20.77 $\pm$ 16.96                | 23.23 $\pm$ 10.81                |                |
|                                         | $T_{2\text{h}}$   | 23.64 $\pm$ 10.47                 | 21.14 $\pm$ 7.51                | 28.74 $\pm$ 13.37                | 26.17 $\pm$ 14.98                |                |
|                                         | $T_{24\text{h}}$  | 25.34 $\pm$ 8.09                  | 17.03 $\pm$ 6.29                | 18.58 $\pm$ 7.74                 | 21.54 $\pm$ 8.59                 |                |
| Nitrotyrosine (nmol L $^{-1}$ )         | $T_{\text{rest}}$ | 76.4 $\pm$ 30.9                   | 70.0 $\pm$ 19.9                 | 71.0 $\pm$ 24.7                  | 89.3 $\pm$ 30.1                  | *              |
|                                         | $T_{\text{ex}}$   | 90.9 $\pm$ 42.3                   | 100.5 $\pm$ 32.9                | 111.0 $\pm$ 39.3                 | 95.8 $\pm$ 57.7                  |                |
|                                         | $T_{1\text{h}}$   | 108.6 $\pm$ 17.7                  | 126.1 $\pm$ 35.5*               | 125.4 $\pm$ 29.3*                | 89.8 $\pm$ 24.8                  |                |
|                                         | $T_{2\text{h}}$   | 100.0 $\pm$ 29.3                  | 98.9 $\pm$ 34.0                 | 102.8 $\pm$ 40.5                 | 78.1 $\pm$ 32.3                  |                |
|                                         | $T_{24\text{h}}$  | 73.1 $\pm$ 31.7                   | 46.3 $\pm$ 24.3                 | 53.0 $\pm$ 35.5                  | 73.9 $\pm$ 35.8                  |                |

Values are expressed as means  $\pm$  SD. AOPP, advanced oxidation proteins products; HbAA-N $\alpha$ T, control healthy subjects (without sickle cell trait and  $\alpha$ -thalassaemia); HbAA- $\alpha$ T,  $\alpha$ -thalassaemic subjects (without sickle cell trait); HbAS-N $\alpha$ T, sickle cell trait carriers (without  $\alpha$ -thalassaemia); HbAS- $\alpha$ T,  $\alpha$ -thalassaemic sickle cell trait carriers; MDA, Malondialdehyde; NOx, nitric oxide metabolism.

\*Significant differences compared with  $T_{\text{rest}}$  ( $P < 0.01$ ).

<sup>†</sup>Significant differences compared with HbAS-N $\alpha$ T at  $T_{\text{rest}}$  and  $T_{\text{ex}}$  ( $P < 0.05$ ); <sup>§</sup> significant differences compared with HbAA-N $\alpha$ T at  $T_{\text{ex}}$  ( $P = 0.02$ ).

protein carbonyls, expressed as the percentage difference from baseline, were not statistically different among the four groups regardless of the time point. Similar to the two other oxidative stress markers, regardless of the group, protein carbonyl increased at  $T_{\text{ex}}$  ( $+83.7 \pm 108.9\%$ , *time effect*:  $P < 0.01$ ); however, it returns to baseline level during the recovery time points ( $+6.9 \pm 77.9\%$  at  $T_{1\text{h}}$ ,  $-12.1 \pm 11.73\%$  at  $T_{2\text{h}}$  and  $-13.2 \pm 9.3\%$  at  $T_{24\text{h}}$ ,  $P > 0.05$ ).

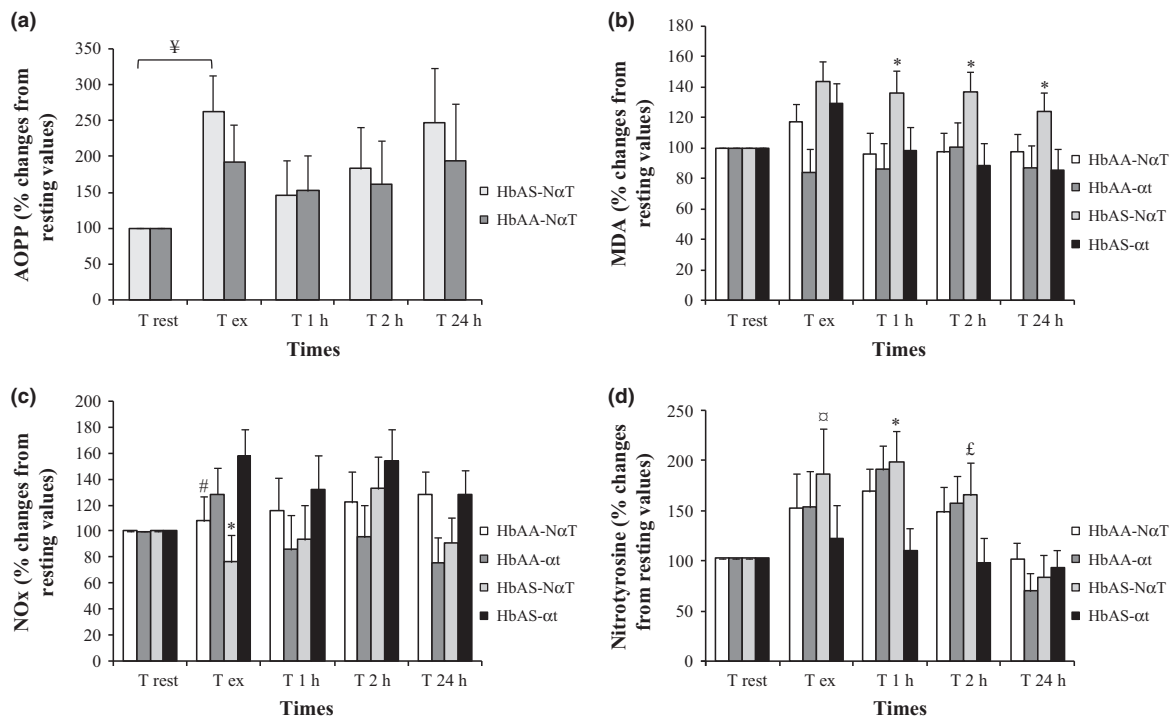
#### Anti-oxidant status in plasma

Table 3 depicts the results of plasma enzymatic anti-oxidant activities and the levels of FRAP. No significant differences were observed at any time among the four groups for SOD, catalase and GPX in both absolute and relative values (i.e. expressed as the change from baseline). However, a time effect in the pooled

population (i.e. independently of HbS and  $\alpha$ -thalassaemia) was noticed, as the activity of SOD was significantly higher at  $T_{\text{ex}}$  and  $T_{1\text{h}}$  in comparison with resting values ( $P < 0.01$ ), whereas data showed lower catalase activities at  $T_{2\text{h}}$  and  $T_{24\text{h}}$  compared with  $T_{\text{rest}}$  ( $P < 0.05$ ). In the same way, GPX was significantly higher at  $T_{\text{ex}}$ ,  $T_{1\text{h}}$  and  $T_{2\text{h}}$  as compared to  $T_{\text{rest}}$  ( $P < 0.001$ ). Finally, FRAP was not modified by the haemoglobin status nor the exercise.

#### Metabolism of nitric oxide and nitrotyrosine

No significant differences were observed in NOx and nitrotyrosine concentrations at rest among the four groups (Table 2). As depicted in the Figure 1(c), NOx at the end of exercise (when expressed in percentage of the baseline value i.e.  $T_{\text{ex}}/T_{\text{rest}}$ ) differed between HbAS- $\alpha$ T and HbAS-N $\alpha$ T ( $P = 0.005$ ). Specifically,



**Figure 1** (a) Plasma AOPP changes immediately after maximal exercise ( $T_{ex}$ ) and during the recovery ( $T_{1h}$ ,  $T_{2h}$  and  $T_{24h}$ ) relative to resting values in HbAA-N $\alpha$ T and HbAS-N $\alpha$ T. Values are presented as means  $\pm$  SE. HbAA-N $\alpha$ T, control healthy subjects (without sickle cell trait and  $\alpha$ -thalassaemia); HbAS-N $\alpha$ T, sickle cell trait carriers (without  $\alpha$ -thalassaemia); \*Significant difference between  $T_{rest}$  and  $T_{ex}$  in HbAS-N $\alpha$ T group ( $P < 0.01$ ). (b, c, d) Plasma malondialdehyde (MDA), NOx and nitrotyrosine changes immediately after maximal exercise ( $T_{ex}$ ) and during the recovery ( $T_{1h}$ ,  $T_{2h}$  and  $T_{24h}$ ) relative to resting values in HbAA-N $\alpha$ T, HbAA- $\alpha$ T, HbAS-N $\alpha$ T and HbAS- $\alpha$ T. Values are presented as means  $\pm$  SE. HbAA-N $\alpha$ T, control healthy subjects (without sickle cell trait and  $\alpha$ -thalassaemia); HbAA- $\alpha$ T,  $\alpha$ -thalassaemic subjects (without sickle cell trait); HbAS-N $\alpha$ T, sickle cell trait carriers (without  $\alpha$ -thalassaemia); HbAS- $\alpha$ T,  $\alpha$ -thalassaemic sickle cell trait carriers; MDA, malondialdehyde; NOx, nitric oxide metabolism. \*Significant differences between HbAS-N $\alpha$ T and all the groups at  $T_{1h}$ ,  $T_{2h}$  and  $T_{24h}$  ( $P < 0.05$ ), #Difference between HbAA-N $\alpha$ T and HbAS-N $\alpha$ T at  $T_{ex}$  ( $P = 0.06$ ),  $^{\text{E}}$ Difference between HbAS-N $\alpha$ T and HbAS- $\alpha$ T groups at  $T_{2h}$  for nitrotyrosine ( $P = 0.07$ ),  $^{\text{D}}$ Difference between HbAS-N $\alpha$ T and HbAS- $\alpha$ T groups at  $T_{ex}$  for nitrotyrosine ( $P = 0.09$ ).

NOx increased in HbAS- $\alpha$ T ( $+57.7 \pm 19.3\%$ ) while it decreased in HbAS-N $\alpha$ T ( $-23.4 \pm 20.6\%$ ). Moreover, analyses of NOx revealed that HbAS-N $\alpha$ T were lower than HbAA-N $\alpha$ T at  $T_{ex}$  ( $P = 0.06$ ).

Conversely, the increase in nitrotyrosine (as expressed in percentage of the baseline value) was significantly lower in HbAS- $\alpha$ T than in HbAS-N $\alpha$ T at  $T_{1h}$  ( $+7.2 \pm 22.2\%$  vs.  $+93.5 \pm 29.3\%$ ,  $P = 0.04$ ; Fig. 1d), and trends also exist for  $T_{ex}$  ( $P = 0.09$ ) and  $T_{2h}$  ( $P = 0.07$ ). Besides, HbAS-N $\alpha$ T had higher levels of nitrotyrosine at  $T_{ex}$  and  $T_{1h}$  compared with  $T_{rest}$  ( $P = 0.07$  and  $P = 0.01$  for  $T_{ex}$  and  $T_{1h}$ , respectively) whereas that HbAA-N $\alpha$ T did not change in response to exercise or during the recovery.

## Discussion

The aim of this study was to determine the consequences of  $\alpha$ -thalassaemia, SCT, and their coexistence

on oxidative stress markers, antioxidant status and NO production at rest and in response to a maximal graded exercise. We found (i) greater lipid peroxidation and protein nitration and (ii) lower NO metabolites in HbAS-N $\alpha$ T than in HbAS- $\alpha$ T in response to exercise. These results suggest that  $\alpha$ -thalassaemia may blunt the elevated post-exercise oxidative stress and associated weakened NO bioavailability observed in SCT carriers.

### Effect of sickle cell trait and/or $\alpha$ -thalassaemia on basal oxidative stress and nitric oxide metabolism

Resting values for oxidative stress, antioxidant markers and NO metabolites were not statistically different between the four groups, except for MDA values that were lower in subjects with SCT than in the dual haemoglobinopathy carriers. The high level of resting MDA in HbAS- $\alpha$ T could be related to the ability of

**Table 3** Plasma antioxidant enzymes activities and FRAP at rest ( $T_{\text{rest}}$ ), immediately after maximal exercise ( $T_{\text{ex}}$ ) and during the recovery ( $T_{1\text{h}}$ ,  $T_{2\text{h}}$  and  $T_{24\text{h}}$ ) in HbAA-N $\alpha$ T, HbAA- $\alpha$ T, HbAS-N $\alpha$ T and HbAS- $\alpha$ T

| Values                                                 | Times             | HbAA-N $\alpha$ T<br>( $n = 14$ ) | HbAA- $\alpha$ T<br>( $n = 8$ ) | HbAS-N $\alpha$ T<br>( $n = 8$ ) | HbAS- $\alpha$ T<br>( $n = 10$ ) | Time<br>effect |
|--------------------------------------------------------|-------------------|-----------------------------------|---------------------------------|----------------------------------|----------------------------------|----------------|
| SOD<br>( $\mu\text{mol mL}^{-1} \text{min}^{-1}$ )     | $T_{\text{rest}}$ | 4.39 $\pm$ 2.53                   | 4.33 $\pm$ 2.18                 | 4.05 $\pm$ 1.95                  | 4.07 $\pm$ 1.09                  |                |
|                                                        | $T_{\text{ex}}$   | 6.00 $\pm$ 1.71                   | 6.73 $\pm$ 2.94                 | 6.90 $\pm$ 9.06                  | 6.91 $\pm$ 3.34                  | *              |
|                                                        | $T_{1\text{h}}$   | 6.09 $\pm$ 1.62                   | 5.49 $\pm$ 1.79                 | 7.48 $\pm$ 1.46                  | 6.32 $\pm$ 1.32                  | *              |
|                                                        | $T_{2\text{h}}$   | 5.62 $\pm$ 2.65                   | 4.45 $\pm$ 2.38                 | 5.04 $\pm$ 2.06                  | 4.24 $\pm$ 1.18                  |                |
|                                                        | $T_{24\text{h}}$  | 5.21 $\pm$ 1.83                   | 4.85 $\pm$ 1.73                 | 5.48 $\pm$ 1.39                  | 3.49 $\pm$ 1.60                  |                |
| Catalase<br>( $\mu\text{mol L}^{-1} \text{min}^{-1}$ ) | $T_{\text{rest}}$ | 4.11 $\pm$ 1.02                   | 4.84 $\pm$ 1.23                 | 4.53 $\pm$ 1.75                  | 5.68 $\pm$ 2.02                  |                |
|                                                        | $T_{\text{ex}}$   | 3.30 $\pm$ 0.91                   | 3.64 $\pm$ 1.61                 | 3.78 $\pm$ 0.68                  | 4.83 $\pm$ 1.45                  |                |
|                                                        | $T_{1\text{h}}$   | 4.75 $\pm$ 1.29                   | 4.35 $\pm$ 1.39                 | 4.13 $\pm$ 0.99                  | 5.19 $\pm$ 1.12                  |                |
|                                                        | $T_{2\text{h}}$   | 4.11 $\pm$ 1.13                   | 3.78 $\pm$ 0.94                 | 3.75 $\pm$ 1.11                  | 3.67 $\pm$ 1.24                  | *              |
|                                                        | $T_{24\text{h}}$  | 3.61 $\pm$ 0.80                   | 3.54 $\pm$ 0.52                 | 3.54 $\pm$ 0.67                  | 3.77 $\pm$ 0.48                  | *              |
| GPX ( $\mu\text{mol L}^{-1} \text{min}^{-1}$ )         | $T_{\text{rest}}$ | 43.6 $\pm$ 31.7                   | 43.3 $\pm$ 19.9                 | 54.2 $\pm$ 31.6                  | 44.1 $\pm$ 7.7                   |                |
|                                                        | $T_{\text{ex}}$   | 111.1 $\pm$ 19.2                  | 131.3 $\pm$ 17.4                | 110.8 $\pm$ 13.0                 | 96.6 $\pm$ 47.5                  | *              |
|                                                        | $T_{1\text{h}}$   | 108.3 $\pm$ 16.5                  | 110.8 $\pm$ 21.8                | 95.9 $\pm$ 17.6                  | 98.9 $\pm$ 24.8                  | *              |
|                                                        | $T_{2\text{h}}$   | 83.5 $\pm$ 31.0                   | 87.7 $\pm$ 19.5                 | 69.4 $\pm$ 15.8                  | 73.5 $\pm$ 19.1                  | *              |
|                                                        | $T_{24\text{h}}$  | 41.7 $\pm$ 24.6                   | 39.2 $\pm$ 26.1                 | 58.2 $\pm$ 11.2                  | 55.5 $\pm$ 26.5                  |                |
| FRAP ( $\mu\text{mol L}^{-1}$ )                        | $T_{\text{rest}}$ | 783 $\pm$ 306                     | 887 $\pm$ 151                   | 768 $\pm$ 45                     | 809 $\pm$ 184                    |                |
|                                                        | $T_{\text{ex}}$   | 728 $\pm$ 196                     | 692 $\pm$ 159                   | 674 $\pm$ 197                    | 680 $\pm$ 269                    |                |
|                                                        | $T_{1\text{h}}$   | 883 $\pm$ 225                     | 725 $\pm$ 140                   | 742 $\pm$ 176                    | 745 $\pm$ 177                    |                |

Values are expressed as means  $\pm$  SD. FRAP, ferric reducing antioxidant power; GPX, glutathione peroxidase; HbAA-N $\alpha$ T, control healthy subjects (without sickle cell trait and  $\alpha$ -thalassaemia); HbAA- $\alpha$ T,  $\alpha$ -thalassaemic subjects (without sickle cell trait); HbAS-N $\alpha$ T, sickle cell trait carriers (without  $\alpha$ -thalassaemia); HbAS- $\alpha$ T,  $\alpha$ -thalassaemic sickle cell trait carriers; SOD, superoxide dismutase.

\*Significant differences compared with  $T_{\text{rest}}$  ( $P < 0.05$ ).

lipid peroxidation products to regulate and modulate antioxidant cell signalling and gene expression (Niki, 2009) and could explain their lower oxidative stress in response to exercise. This mechanism should induce an improvement in the antioxidant enzyme efficiency in HbAS- $\alpha$ T. Unfortunately, the plasma antioxidant markers did not revealed significant difference between the four groups. Nevertheless, according to Ginsburg *et al.* (2011), the plasma alone does not represent the whole oxidant-scavenging abilities of the blood because the RBCs have also a significant impact on the antioxidant capacity. Thus, our results could be explained by the assessment of antioxidant enzymatic activities only in the plasma and not in the RBCs.

In addition, the lack of a difference between HbAA- $\alpha$ T and HbAA-N $\alpha$ T for MDA resting concentrations contrasts with the study of Cheng *et al.* 2004 who depict increased resting TBARS in patients with  $\alpha$ -thalassaemia compared with control subjects. This discrepancy could be explained by the different types of thalassaemic deletion ( $\alpha/\alpha^{3,7}$  in our population vs. homozygous or Southeast-Asian deletion in Cheng *et al.* 2004). Nevertheless, neither HbAS-N $\alpha$ T nor HbAS- $\alpha$ T values were different from those of control subjects suggesting that these MDA concentrations could be considered as normal in both groups.

#### Exercise-induced oxidative stress and nitric oxide metabolism in $\alpha$ -thalassaemic subjects

As hypothesized, lipid and protein oxidation in response to exercise were not enhanced in  $\alpha$ -thalassaemic subjects compared with controls (HbAA-N $\alpha$ T). Similarly, NOx and antioxidant enzymes were not different between these two groups. These results support that heterozygous  $\alpha^{3,7}$  deletion might be considered as asymptomatic with regard to oxidative stress.

#### Exercise-induced oxidative stress and nitric oxide metabolism in sickle cell trait carriers

Contrary to the three other groups, MDA levels (relative to resting values) remained elevated in SCT carriers during the entire period of recovery following the maximal incremental exercise (Fig. 1b). Moreover, independent of the  $\alpha$ -thalassaemia genetic disorder, only plasma level of MDA increased in SCT carriers at  $T_{\text{ex}}$ . In addition, there was a very strong trend ( $P = 0.06$ ) for SCT carriers (HbAS-N $\alpha$ T group) to express lower levels of NOx (Fig. 1c). Finally, on the contrary to the control subjects, higher levels of nitrotyrosine were found in SCT carriers at the end of exercise ( $P = 0.07$ ) and after 1 h of recovery ( $P = 0.01$ ) (Fig. 1d). These results support the idea

that i) oxidative stress is likely higher and ii) NO metabolism could be altered in SCT carriers after maximal incremental exercise compared with HbAA-N $\alpha$ T subjects. However, this latter hypothesis should be moderated because we only measured systemic NO metabolites that may not reflect intracellular NO. Nevertheless, it was shown that a decrease in plasma NOx is highly related to physiological evidence of NO-dependent endothelial dysfunction (Kleinbongard *et al.* 2006). These results are in accordance with those of Das *et al.* 1993 who reported a trend towards an increased lipid peroxidation in erythrocytes of SCT carriers in response to a similar type of exercise. Those results also tend to support our hypothesis (see Introduction) that a high intensity exercise is required to significantly disturb ROS and NO metabolism in SCT carriers. Furthermore, the lack of a change in the antioxidant defences in comparison with control subjects suggests that the antioxidant/prooxidant balance is disturbed by an overgeneration of ROS in the SCT carriers.

#### *Effect of $\alpha$ -thalassaemia on NOx, Nitrotyrosine levels, lipid peroxidation and protein oxidation in sickle cell trait carriers*

In accordance with our first hypothesis, the higher NOx at the end of exercise and the lower nitrotyrosine levels at T<sub>1h</sub> in HbAS- $\alpha$ T compared with HbAS-N $\alpha$ T suggest that the presence of  $\alpha$ -thalassaemia in the SCT carriers could dampen the deleterious effects associated with SCT on NO metabolism and the potential subsequent endothelial dysfunction as observed in previous studies (Wood *et al.* 2006). Moreover, the trend followed by nitrotyrosine values at T<sub>ex</sub> and T<sub>2h</sub> strengthened this idea. This positive effect might be related to the fact that  $\alpha$ -thalassaemia decreases the HbS content in the RBCs of SCT carriers (Table 1). The lower HbS content would then provide a relative protection against sickling (Harkness 1989), which was reported to increase ROS generation (Hebbel *et al.* 1982). Moreover, a reduced ROS production would restrict NO from reacting with superoxide anion (O<sub>2</sub><sup>•-</sup>) to form peroxynitrite (ONOO<sup>-</sup>), a powerful oxidant (Singh *et al.* 2002), thereby improving the bioavailability of NO. In the context of SCT, this higher NO metabolites content, which could suggest an improvement of NO metabolism because of the presence of  $\alpha$ -thalassaemia, seems particularly important at T<sub>ex</sub> because it may limit the possible vasoconstriction associated with NO decrease and eventual endothelial dysfunction. In accordance with this hypothesis, it is interesting to note that  $\alpha$ -thalassaemia blunts lipid peroxidation during the recovery in SCT subjects. Indeed, Figure 1(b) shows

that while MDA levels remain elevated in SCT carriers during the entire period of recovery following the maximal incremental exercise (T<sub>1h</sub>, T<sub>2h</sub> and T<sub>24h</sub>), MDA levels in subjects with the dual haemoglobinopathy mimic those of control subjects.

The results of oxidative stress, NOx and nitrotyrosine changes in response to acute physical exercise corroborate previous findings that showed that the  $\alpha$ -thalassaemia in SCT carriers might have a protective role against the exercise-induced haemorheological disturbances (Monchanin *et al.* 2005) and endothelial activation (Monchanin *et al.* 2007). These similar beneficial effects of  $\alpha$ -thalassaemia seem to imply that oxidative stress could be associated with adhesion and inflammation as it was shown in SCD (Wood & Granger 2007). Oxidative stress is also recognized as an important feature in the pathogenesis of SCD via its implication in haemolysis and vaso-occlusive crisis (Nur *et al.* 2011, Chirico & Pialoux 2012). Indeed, several mechanisms contribute to the production of ROS in patients with SCD including the higher auto-oxidation of the sickle haemoglobin (HbS). As our results suggest that alpha-thalassaemia could limit the negative effects of ROS in SCT carriers, the coexistence of these both genetic defects could have a protective effect.

#### *Study limitations*

The relatively small number of subjects per group (14 in HbAA-N $\alpha$ T and eight in HbAS-N $\alpha$ T) in this study did not allow us to reach the significant difference threshold for NOx, MDA, nitrotyrosine and AOPP in T<sub>ex</sub>. However, MDA concentration was significantly higher in HbAS-N $\alpha$ T than in HbAA-N $\alpha$ T throughout all recovery time points (see Fig. 1b). In addition, a power calculation with an alpha set at 0.05 and power set at 0.80 gave a sample size of 11, 16, 20 and 30 subjects per group for NOx, MDA, nitrotyrosine and AOPP, respectively, at the end of exercise.

#### *Conclusion*

Similarly to what was previously shown with exercise-induced inflammation (Monchanin *et al.* 2007), the coexistence of  $\alpha$ -thalassaemia in SCT subjects seems to have a beneficial impact on the elevated oxidative stress and NO activity in SCT carriers. Indeed, the present findings show that subjects with both genetic abnormalities, SCT and  $\alpha$ -thalassaemia had lower levels of lipid peroxidation and protein nitration and greater NO metabolites content in response to acute physical exercise. As a consequence, these results suggest that SCT patients with  $\alpha$ -thalassaemia may be more protected against pathophysiological

mechanisms induced by maximal exercise. Although we found significant effects of  $\alpha$ -thalassaemia on systemic oxidative stress and NO metabolism when expressed from relative changes from baseline, further studies should include larger population to strength and confirm our results.

### Conflict of interest

Camille Faës: none declared, Cyril Martin: none declared, Erica N. Chirico: none declared, Léonard Féasson: none declared, Samuel Oyonno-Enguelle: none declared, Hervé Dubouchaud: none declared, Alain Francina: none declared, Patrice Thiriet: none declared, Vincent Pialoux: none declared, Laurent Messonnier: none declared.

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**IX.1e In vivo cardiac anatomical and functional effects of wheel running in mice by magnetic resonance imaging**



## *In vivo* cardiac anatomical and functional effects of wheel running in mice by magnetic resonance imaging

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### Abstract

Physical activity is frequently used as a strategy to decrease pathogenesis and improve outcomes in chronic pathologies such as metabolic or cardiac diseases. In mice, it has been shown that voluntary wheel running (VWR) could induce an aerobic training effect and may provide a means of exploring the relationship between physical activity and the progression of pathology, or the effect of a drug on locomotor activity. To the best of our knowledge, *in vivo* magnetic resonance imaging (MRI) and other non-invasive methods had not been investigated for training evaluation in mice; therefore, it was proposed to test an MRI method coupled with a cardiorespiratory gating system on C57Bl/6 mice for *in vivo* heart anatomical and functional characterization in both trained and untrained animals. Twenty mice were either assigned to a 12-week VWR program or to a control group (CON – no wheel in the cage). At week 12, MRI scans showed an increase in the left ventricular (LV) wall mass in the VWR group compared with the CON group. The *ex vivo* measurements also found an increase in the heart and LV weight, as well as an increase in oxidative enzyme activities (i.e. cytochrome c oxidase [COx] in the soleus). In addition, correlations have been observed between *ex vivo* LV/body weight ratio, COx activity in the soleus and *in vivo* MRI LV wall mass/body weight. In conclusion, mouse cardiac MRI methods coupled with a cardiorespiratory gating system are sufficiently effective and feasible for non-invasive, training-induced heart hypertrophy characterization, and may be used for longitudinal training level follow-up in mouse models of cardiovascular and metabolic diseases.

**Keywords:** enzymology, MRI heart function, mouse, physical training, imaging markers, left ventricular hypertrophy

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### Introduction

Magnetic resonance imaging (MRI) is an increasingly used imaging modality in animal experiments as it offers a non-invasive option for observation of tissues and cells. For example, MRI has been used for *in vivo* organ characterization (heart, spleen, brain)<sup>1</sup> as well as for cellular observation using macrophage or adhesion molecule nanoparticle targeting strategies.<sup>2</sup> The key benefits of non-invasive MRI protocols include (1) a reduction in the number of animals used and sacrificed due to the possibility of longitudinal follow-up; (2) the option of using the same animal as a control; and (3) a reproducible and sufficiently sensitive method for the simultaneous assessment of multiple markers.<sup>2,3</sup>

The purpose of this study was to perform an *in vivo* MRI heart characterization in mice in order to evaluate their

physical training level, which is known to be associated with an increase in cardiac mass. Indeed, the prevalence of chronic pathologies such as diabetes, metabolic disease, heart failure, and cardiovascular disease or neuronal degeneration has led to the addition of regular physical training as an important strategy for the prevention or the treatment of these diseases. Physical activity is known to elicit physical and metabolic adaptations in cardiac<sup>4</sup> and skeletal muscle,<sup>5</sup> and may therefore decrease the pathogenesis of these diseases.<sup>6–13</sup>

Moreover, exercise activities are used in clinical trials as a readout parameter to explore the benefits of novel pharmacological or molecular agents for the treatment of heart failure or diabetes.<sup>14,15</sup> Therefore, the preclinical development of such agents will benefit significantly from the



development of a non-invasive MRI preclinical protocol to evaluate therapies *in vivo* in a serial manner.

Murine models are frequently used to study pathophysiology or the effects of therapeutic strategies in heart failure,<sup>16,17</sup> metabolic and genetic diseases,<sup>18</sup> and voluntary wheel running (VWR) is among the most commonly used exercise training procedure in these animals. Its effects are characterized by cardiac hypertrophy<sup>19</sup> and an increase in some oxidative (COx – cytochrome *c* oxidase)<sup>20–22</sup> or Krebs cycle (CS – citrate synthase; SDH – succinate dehydrogenase)<sup>4,20–25</sup> enzyme activities. In this context, it may be interesting to develop a non-invasive, robust and reproducible marker of the training level in mice.

To evaluate the training level in these animals, the authors rely on activity data (such as mean daily or weekly running distance and/or time) or *ex vivo* measurements (such as muscle enzyme activities or heart weight). To our knowledge, no *in vivo* procedure has ever been described to determine the physiological impact of a training program in mice. MRI offers the possibility of examining the heart *in vivo*, but it is associated with technical difficulties in small animals because of the high frequency of respiratory movements. In the present study, we propose to validate an *in vivo* method in order to evaluate the training level in mice by measuring the anatomical and functional left ventricular (LV) parameters. For this purpose, we used a non-invasive MRI method coupled with a cardiorespiratory gating system in order to acquire images during the expiration phase of the respiratory cycle and prevent artifacts due to respiratory perturbation.

## Methods

### Animals

To avoid a gender effect, 12-week-old male C57BL/6 mice ( $n = 20$ ) (Charles River, L'Arbresle, France) were randomly assigned to a control group (CON;  $n = 10$ ) or VWR group ( $n = 10$ ). After reception and one week of acclimation, mice were assigned to housing at two per cage with or without running wheels. After a two-week wheel adaptation period, mice were assigned to an individual cage. All animals were maintained on a 12 h light–dark cycle and were supplied with food and water *ad libitum*. One week before the dissection, a cardiac MRI was performed on six mice per group. Body weight, food and water consumption were recorded weekly. The ACSM animal care standards for experimental procedures were followed and all animal protocols were approved by the regional animal care committee (Rhones-Alpes, France).

### Voluntary wheel-running protocol

All animal houses were equipped with a mouse 12.5-cm metal wheel (Hagen-61700; Montreal, QC, Canada). The number of completed revolutions was monitored by a magnetic switch affixed to each wheel and data were captured using a digital magnetic counter (model BC906; Sigma Sport, Neustadt, Germany). During the 12-week experimental period, the total running distance, total running time and

average speed were recorded before the counter was reset each week.

### *In vivo* cardiac MRI

Imaging was performed on a 4.7 Tesla Oxford magnet interfaced to a Bruker console (Bruker Biospin GmbH, Rheinstetten, Germany) equipped with a 10-cm diameter actively shielded gradient set (250 mT/m). A homemade Alderman and Grant volume coil (30 mm length and 26 mm inner diameter) was used to obtain optimal RF homogeneity over the volume of interest.

Eleven weeks after the beginning of the running protocol, cardiac MRI was performed on 12 mice randomly selected (6 VWR and 6 CON) using a homemade double cardiorespiratory gating system following the procedure of Alsaïd *et al.*<sup>26</sup> Mice were anesthetized with continuously inhaled isoflurane at 1.5–2% in ambient air for maintaining a stable respiratory rate. The body temperature was maintained in the magnet using a circulating water heating blanket (37°C). Following a tripilot gradient-echo image, a 2D-FLASH sequence was used to acquire two long-axis slices (in the coronal and sagittal planes) using the following parameters: TR/TE = 124/4 ms, matrix = 256 × 256, field of view (FOV) = 35 × 35 mm, slice thickness = 1 mm and number of average (NAV) = 2. Perpendicular to the long axis, a 2D-FLASH sequence was used to acquire short-axis cine images (5–6 contiguous slices covering the entire left ventricle): TR/TE = 10/3 ms, flip angle = 20°, matrix = 128 × 128, FOV = 20 × 20 mm, slice thickness = 1 mm, NAV = 8 and 15–17 frames (images) were constructed to cover each phase of the entire cardiac cycle (R–R) of the animal.

### MR image analysis

All images were analyzed using Image J Software (Wayne Rasband, National Institute of Mental Health, Bethesda, MD, USA). LV function was evaluated using short-axis images in end diastole and end systole. The endocardial (LV inner border) and epicardial borders (LV outer border) were manually traced and end-diastolic volume (EDV), end-systolic volume (ESV), stroke volume (SV), ejection fraction (EF) and LV myocardial mass (LV wall mass) were computed from the traced borders. To calculate the LV myocardial mass, the end-diastolic myocardial volume (EDV outer border minus EDV inner border) was multiplied by the specific density of the myocardium (1.05 g/cm<sup>3</sup>). The LV remodeling index (LV myocardial mass/EDV) was calculated in order to determine the pattern of ventricular remodeling in the two groups. An increased remodeling index is consistent with concentric hypertrophy, whereas a reduced remodeling index is indicative of isolated cavity dilation.<sup>27</sup>

### Muscle isolation and heart weight

After waiting one week for the imaged mice to completely recover from anesthesia, all the mice were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg; Doletal®, Vétquinol, Lure, France) and transcardially

perfused for 70 s with 9‰ NaCl (Sigma, St Louis, USA). The heart was removed, rinsed in saline, dried with a paper towel and weighed, and the left ventricle was dissected and weighed separately.

Soleus and tibialis anterior (TA) muscles were dissected, weighed and immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

### Mitochondrial enzymatic activities

Lower limb muscles are among the most relevant to evaluate the physical training response on mitochondrial enzymatic activities.<sup>20,28,29</sup> Thus, one TA or two soleus muscles were cut into thin pieces and homogenized on ice at 10% with a buffer (225 mmol/L mannitol; 75 mmol/L sucrose; 10 mmol/L Tris-HCl, 0.1 mmol/L EDTA, pH 7.2). Homogenates were then centrifuged for 20 min at 2500g ( $4^{\circ}\text{C}$ ). Supernatants were frozen at  $-80^{\circ}\text{C}$ . Protein concentrations were determined spectrophotometrically (Biophotometre, Eppendorf, Germany) using a BCA kit (Sigma).

### COx activity

The complex IV activity was measured in the soleus and TA, as previously described,<sup>30</sup> by spectrophotometric observation (at 550 nm) of the cytochrome *c* (C-7752; Sigma) oxidation kinetics.

### CS activity

CS activity was measured in the soleus and TA by the method of Shepherd and Garland.<sup>31</sup> Briefly, the citrate synthesis rate from acetyl coenzyme A and oxaloacetate was determined according to a coupling reaction between coenzyme A and DTNB (5,5'-dithiobis [2-nitrobenzoic acid]). This coupling reaction was spectrophotometrically measured at 412 nm during 45 s.

### SDH activity

SDH activity was measured in the soleus and TA by DCIP reduction kinetics (2,6-dichloroindophenol; D1878; Sigma) at 600 nm as previously described.<sup>30</sup>

All the enzymatic activities were measured with a spectrophotometer (InfiniteM200; Tecan, Männedorf, Switzerland) at  $37^{\circ}\text{C}$  and expressed in nanomoles of product formed per minute and per milligram of protein. All products were purchased from Sigma.

### Statistical analysis

All statistical analyses were performed using STATISTICA (Tulsa, OK, USA) on six mice per group for the MRI parameters and on 10 mice per group for the *ex vivo* parameters. The data are expressed as mean  $\pm$  SE. To determine the significance of parameter changes, the Mann-Whitney *U* test was performed between the CON and VWR groups. Spearman correlation coefficients were used to describe the linear association between variables. Probability (*P*) values of  $<0.05$  were considered statistically significant.

## Results

### Animal characteristics and running wheel activity

Quantitative results for the food and water consumptions and the exercise activities are summarized in Table 1. Weekly consumption of food and water was significantly higher (+22.9% and +18.7%, respectively) in the VWR group compared with the CON group. However, as all weights were similar in both groups at the beginning of the study (CON:  $23.47 \pm 0.67$  g and VWR:  $23.93 \pm 0.41$  g), this increase was not associated with any changes in body weight in either group. Mice housed in a cage equipped with a running wheel ran an average distance of  $37.46 \pm 2.35$  km per week over 35 h 19 min  $\pm$  6 h 22 min.

The exercise phenotype, including the average distance run over the 12 weeks in the VWR group, is shown in Figure 1. Mice reached a maximal distance during the third week (+26% compared with the average distance of the 12 weeks) once isolated in an individual cage. Through the fourth and fifth week, the running distance was still greater than the average distance (+16.4% and +9.6%, respectively, compared with the average distance of the 12 weeks). In the sixth week, the running distance plateaued for three weeks (distance run is equal to the average distance) and was significantly lower than the distance run at week 3. Finally, the shortest running distance was reached in the 11th week ( $-49\%$  compared with the average distance of the 12 weeks).

### Heart parameters

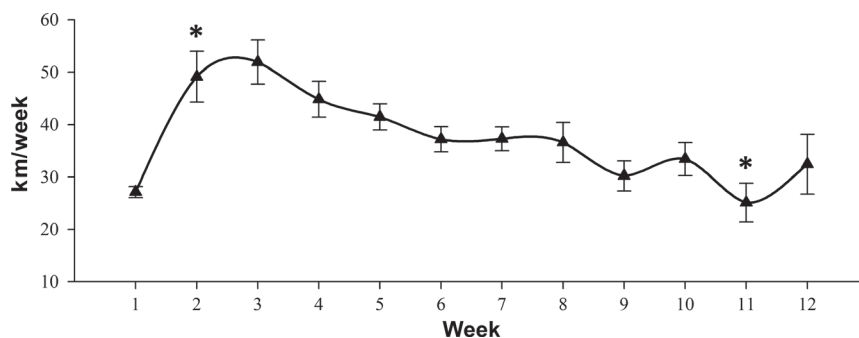
During MRI, the heart rate was maintained and no difference was observed between the CON and VWR groups ( $414.1 \pm 10.4$  and  $417.2 \pm 16.2$  bpm respectively;  $P = 0.62$ ). Figure 2a depicts short-axis MR images acquired in the center of the heart in CON and VWR mice. Quantitative

**Table 1** Animals characteristics in control (CON) or voluntary wheel running (VWR) mice during the 12-week protocol

|                            | CON (n = 10)     | VWR (n = 10)            | P       |
|----------------------------|------------------|-------------------------|---------|
| Body weight (g)            | $26.66 \pm 0.36$ | $26.95 \pm 0.29$        | 0.680   |
| Water consumption/week (g) | $32.00 \pm 1.03$ | $41.53 \pm 1.22$        | 0.0003* |
| Food consumption /week(g)  | $31.06 \pm 0.57$ | $38.21 \pm 1.11$        | 0.0002* |
| Distance run/week (km)     | –                | $37.46 \pm 2.35$        | –       |
| Time run/week (h:min:s)    | –                | $35:19:14 \pm 06:22:09$ | –       |

Values are means  $\pm$  SE

\* $P < 0.05$  (VWR versus CON)



**Figure 1** Distance ran per week in voluntary wheel running C57Bl/6 mice ( $n = 10$ ). Values are means  $\pm$  SE. \* $P < 0.05$  (versus previous week)

MRI results for the cardiac morphometric and functional indices are summarized in Figure 2b. The LV wall mass and LV wall mass/body weight (BW) ratio were significantly higher in the VWR group ( $92.11 \pm 3.06$  mg and  $3.44 \pm 0.13$  mg/g, respectively) compared with the control mice ( $77.61 \pm 4.58$  mg and  $2.96 \pm 0.17$  mg/g,  $P < 0.05$ ). The EDV, ESV and SV also presented higher values for the running wheel mice compared with the control mice (+20.32% for EDV; +44.9% for ESV; +17.1% for SV;  $P < 0.05$ ). However, the EF (Figure 2b,  $P = 0.12$ ) and the LV remodeling index ( $1.91 \pm 0.11$  for CON mice and  $1.80 \pm 0.08$  g/mL for VWR mice,  $P = 0.52$ ) did not differ among the two groups.

Postmortem analysis, as presented in Figure 3, showed significant increases in the heart weight to body weight ratio (HW/BW) and in the LV weight to body weight ratio (LVW/BW) in the VWR group compared with the CON group (+12.4% and +20.8%, respectively).

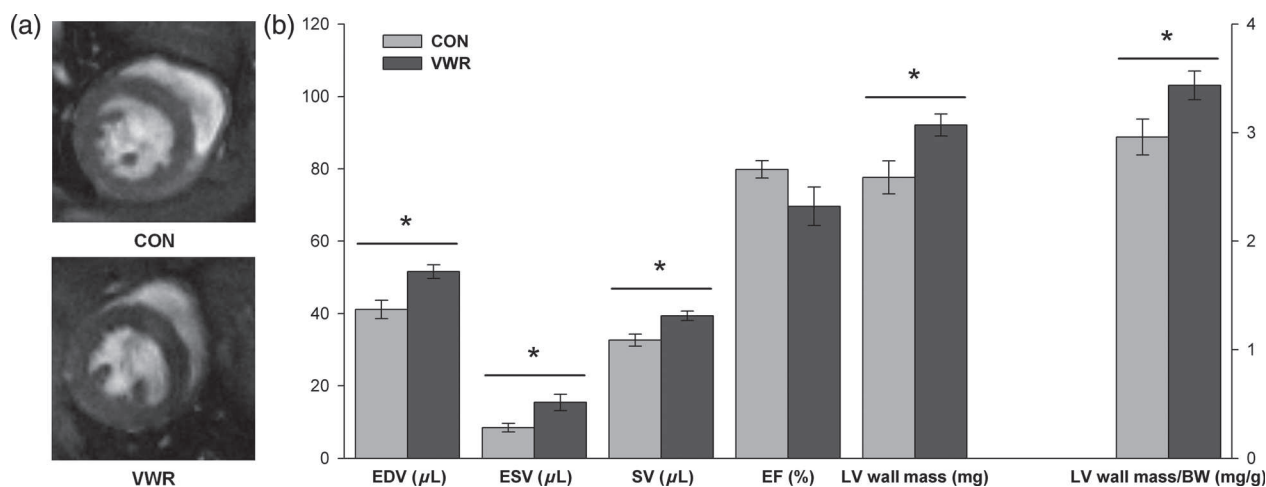
### Muscle characteristics and enzyme activities

Table 2 shows the skeletal muscle enzyme activities and weight from the soleus and TA in the two groups. Measurements were performed on 10 mice per group,

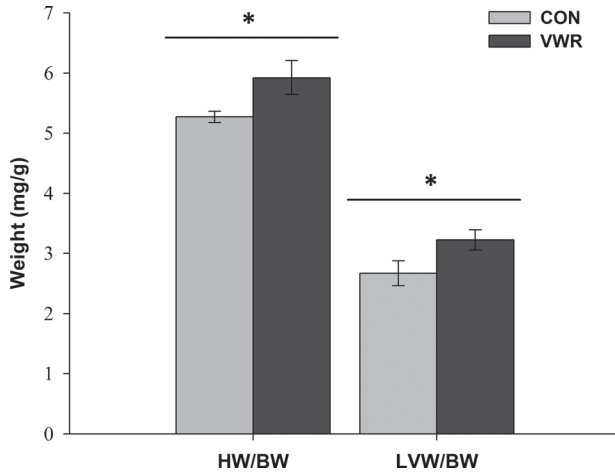
except for the exclusion of one CON soleus muscle due to a technical problem during the dissection. An increase in muscle weight and the muscle weight/body weight ratio was observed in the VWR group compared with the CON group in the soleus as well as in TA ( $P < 0.05$ ; Table 2). Furthermore, whereas no differences in SDH activity in both the muscles and in COx activity in the TA were observed between the two groups, VWR mice exhibited higher COx activity in the soleus compared with the CON mice (+28.8%;  $P < 0.05$ ).

### Relationships between MRI and biochemical parameters

The relationships between the enzymology, MRI heart parameters (LV wall mass/BW and EDV) and heart, and muscle postmortem parameters (LVW/BW and soleus weight/BW) are shown in Figure 4. A positive correlation was observed between the MRI LV wall mass and the post-mortem LVW ( $P = 0.719$  and  $0.005$ ; data not shown) as well as between the MRI LV wall mass/BW ratio and the post-mortem LVW/BW ( $r = 0.671$ ;  $P = 0.017$ ; Figure 4a). The MRI EDV tended to be related to the postmortem soleus weight/BW ratio ( $r = 0.663$ ;  $P = 0.026$ ; Figure 4b) and



**Figure 2** Representative short-axis midventricular magnetic resonance images acquired in control (CON;  $n = 6$ ) and voluntary wheel running (VWR;  $n = 6$ ) mice. The VWR heart in (a) is characterized by an enlarged LV chamber volume and a greater myocardial mass (b) ( $92.11 \pm 3.06$  mg for the VWR group compared with  $77.61 \pm 4.58$  mg for the CON group). Values are means  $\pm$  SE. VWR, voluntary wheel running; EDV, end-diastolic LV chamber volume; ESV, end-systolic LV chamber volume; SV, stroke volume; EF, ejection fraction; LV, left ventricle; BW, body weight. \* $P < 0.05$  (VWR versus CON)



**Figure 3** Postmortem heart and LV weights in control (CON;  $n = 10$ ) or voluntary wheel running (VWR;  $n = 10$ ) C57Bl/6 mice. Values are means  $\pm$  SE. HW/BW, heart weight/body weight; LVW/BW, left ventricular weight/body weight. \* $P < 0.05$  (CON versus VWR)

additionally, the COx activity in the soleus and was statistically related to the MRI LV wall mass/BW ( $r = 0.718$ ;  $P = 0.013$ ; Figure 4c) and to the postmortem LVW/BW ratio ( $r = 0.556$ ;  $P = 0.016$ ; Figure 4d).

## Discussion

The purpose of the study was to validate an *in vivo* cardiac MRI method in order to evaluate the training level of mice. Following the 12-week running wheel session, it was observed that exercise training induced physiological modifications in heart and lower limb muscles, and that MRI was sufficiently sensitive to detect these variations in the heart. Indeed, it was observed that there was: (1) an increase in the postmortem whole heart, left ventricle, soleus and TA weights; (2) an increase in MRI heart parameters such as LV wall mass, EDV, ESV and SV; (3) an increase in enzymatic activities such as that of COx; and (4) COx activity in the soleus and LVW/BW were correlated to LV wall mass/BW obtained with MRI.

As cardiovascular adaptations are among the most important responses to physical activity,<sup>4,13,32</sup> we chose to evaluate heart anatomical and functional changes using proton MRI.

MRI offers the possibility of examining *in vivo* organs but is often associated with additional technical difficulties in mice, as opposed to rats,<sup>33</sup> because of the frequency of their cardiorespiratory movements. The double realtime cardiorespiratory gating system used here and developed by Sabbah *et al.*<sup>1</sup> and Alsaïd *et al.*,<sup>26</sup> allowed us to reconstruct high-resolution heart images throughout the cardiac revolution.<sup>1</sup> Other options using self-gated MRI methods<sup>34</sup> and parallel imaging techniques<sup>35</sup> may further facilitate manipulation for longitudinal MRI follow-up in mice.

Our results show that the LV wall mass measured with MRI was increased in VWR mice compared with CON mice and was related to both the COx activity in the soleus and to the postmortem LVW/BW ratio (Figures 4a–c). In addition, the cardiac work capacity notably evaluated by MRI (EDV) was related to the soleus weight and the soleus weight/BW ratio (Figure 4b). The increase in COx activity and organ weight (heart, LV, soleus and TA) are often used as markers of the training level in many models.<sup>4,18,19,32,36,37</sup> Therefore, the existence of a significant correlation between the postmortem markers and the *in vivo* MRI measurement supports cardiac MRI as a sensible choice of technique for the detection of cardiac adaptations to exercise training in mice. Indeed, the MRI LV mass was slightly but not significantly higher than the postmortem LV mass ( $84.9 \pm 3.4$  and  $81.7 \pm 6.1$  mg, respectively), as was already observed in other animal models.<sup>38,39</sup>

The first major interest in using this technique is that it allows for the evaluation of the cardiovascular effects of exercise training in mice without euthanasia, making it possible to continue the training protocol if adaptations are not significant. Adaptation to exercise training is known to differ between mouse strains,<sup>40,41</sup> so the present MRI technique could be applied to determine the unknown adaptive capacity of numerous cardiovascular transgenic mouse models to habitual exercise. It can also be used to explore, *in vivo*, the benefits of novel pharmacological agents on mouse running capacity. Another interest is that this method makes it possible to decrease the number of animals sacrificed per study as observed by us and others.<sup>3,33–35</sup> Whereas 10 animals per group were necessary to obtain a significant difference between the VWR and the CON mice using *ex vivo* measurements (enzymology, organ weight), only six animals per group were used according to

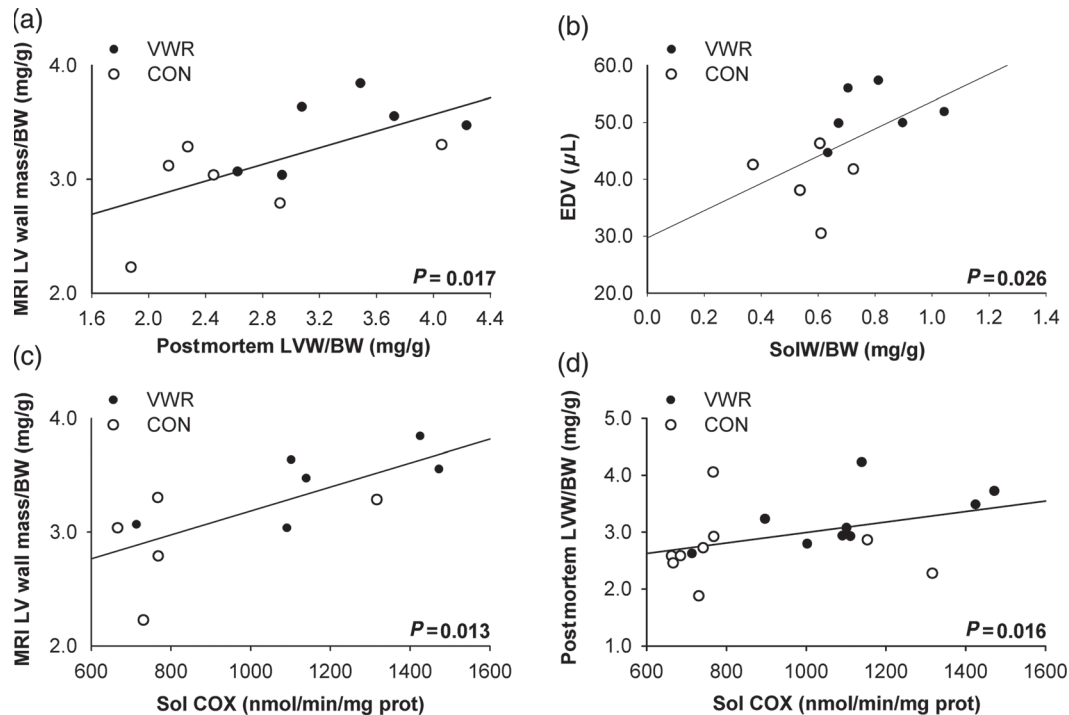
**Table 2** Soleus and tibialis anterior (TA) mass and enzymatic characteristics in control (CON) or voluntary wheel running (VWR) C57Bl/6 mice

|                                 |     | CON              | VWR                | % increase | P      |
|---------------------------------|-----|------------------|--------------------|------------|--------|
| Muscle mass (mg)                | Sol | 15.75 $\pm$ 0.79 | 19.61 $\pm$ 1.32   | +19.69%    | 0.013* |
|                                 | TA  | 35.93 $\pm$ 3.15 | 47.94 $\pm$ 4.10   | +25.05%    | 0.044* |
| Ratio muscle mass/BW (mg/g)     | Sol | 0.59 $\pm$ 0.03  | 0.73 $\pm$ 0.05    | +19.35%    | 0.038* |
|                                 | TA  | 1.35 $\pm$ 0.13  | 1.79 $\pm$ 0.16    | +24.39%    | 0.011* |
| COx activity (nmol/min/mg prot) | Sol | 771.7 $\pm$ 56.5 | 1 105.7 $\pm$ 78.7 | +28.8%     | 0.047* |
|                                 | TA  | 637.8 $\pm$ 64.4 | 737.4 $\pm$ 59.3   | +13.5%     | 0.34   |
| CS activity (nmol/min/mg prot)  | Sol | 21.5 $\pm$ 1.5   | 23.6 $\pm$ 3.2     | +4.9%      | 0.33   |
|                                 | TA  | 8.1 $\pm$ 0.3    | 8.4 $\pm$ 0.3      | +3.41%     | 0.46   |
| SDH activity (nmol/min/mg prot) | Sol | 2.37 $\pm$ 0.17  | 3.66 $\pm$ 1.29    | +35.3%     | 0.68   |
|                                 | TA  | 1.16 $\pm$ 0.08  | 1.22 $\pm$ 0.09    | +4.8%      | 0.83   |

BW, body weight; COx, cytochrome c oxidase; CS, citrate synthase; SDH, succinate dehydrogenase; Sol: Soleus (CON:  $n = 9$  and VWR:  $n = 10$ ); TA: tibialis anterior (CON and VWR:  $n = 10$ )

Values are means  $\pm$  SE. \* $P < 0.05$  (CON versus VWR)





**Figure 4** Correlations between parameters measured in control (CON) or voluntary wheel running (VWR) C57Bl/6 mice. (a) MRI LVM/BW related to the postmortem LVW/BW ratio ( $n = 6$ ); (b) end-diastolic volume (LV chamber) related to soleus weight/BW ratio; (c) MRI LVM/BW related to COx activity in soleus muscle; (d) postmortem LVW/BW ratio related to the COx activity in soleus muscle. MRI LVM/BW, magnetic resonance imaging left ventricular wall mass/body weight (CON and VWR:  $n = 6$ ); LVW/BW, left ventricular weight/body weight (CON and VWR:  $n = 10$ ); EDV, end-diastolic volume (CON and VWR:  $n = 6$ ); SolW/BW, soleus weight/body weight (CON:  $n = 9$  and VWR:  $n = 10$ ); Sol COx, cytochrome c oxidase activity in soleus (CON:  $n = 9$  and VWR:  $n = 10$ )

the *in vivo* MRI method. This kind of animal experiment is in accordance with the recommendations of the international animal care committees. Moreover, as six of the 10 mice performed MRI, we further showed that anesthesia had no effect on the enzymatic results, i.e. in each group, there was no significant difference between mice with MRI and those without.

In this study, the use of VWR in mice was chosen because it is a widely used and approved method to obtain a trained mouse model. This voluntary training model is often preferred to swimming exercise or forced treadmill training because the latter is associated with chronic stress including the releasing of stress hormones.<sup>42</sup> Several authors have shown that in free wheel activity, mice run sufficiently to produce physiological adaptations observed with aerobic training, such as cardiac hypertrophy<sup>4,18,19</sup> and better muscle oxidative capacity.<sup>5,20–22,24,25,28,29</sup> This is confirmed in the present study where 12 weeks of physical activity were associated with heart and LV hypertrophy (Figure 2) as well as musculoskeletal changes (Table 2). The left ventricle hypertrophy observed in trained mice was defined as 'symmetric' because the LV remodeling index was similar between the CON and VWR mice. The definition of this characteristic is an increase in cavity dimension and volume accompanied by an increase in thickness and mass of the ventricles without modification of LV geometry.<sup>27</sup> This is in agreement with studies on elite triathletes, soccer players and rowers where there was no difference between their control and trained groups.<sup>27,36,43</sup>

Wheel running in mice was shown to increase muscle mass (TA and Sol), muscle mass/BW ratio and the COx activity in soleus. Because soleus is an oxidative muscle,<sup>44</sup> the increase in COx activity in the soleus implies that the wheel running was responsible for enhancing the efficiency of the COx activity and consequently the oxidative capacity,<sup>30</sup> but not the amount of functional mitochondria. Indeed, CS and SDH, Krebs cycle enzymes which reflect the amount of functional mitochondria, did not increase.<sup>30</sup> The oxidative capacity of the TA does not seem to be influenced by the wheel running either. Our results are in contrast with Allen *et al.*<sup>4</sup> who observed, in the TA, a shift of the muscle fibers from the glycolytic IIb and IId fibers to the more oxidative IIa fibers after only four weeks of wheel running, which should have modified the oxidative capacity of the muscle.<sup>4</sup> These discrepancies could be explained by the average running distance and running intensity. A longer running distance, as presented in the Allen *et al.* study,<sup>4</sup> is sufficient to induce a shift in the TA, while a running intensity inferior to 50% of maximal oxygen consumption, as suggested by Dudley *et al.*,<sup>45</sup> may not elicit an adaptation in mitochondrial content, particularly in fast-twitch like TA. These combined observations may explain our results.

This study had several limitations. First, there were no baseline MRI measurements before the eight weeks of VWR. As C57Bl/6 are inbred, it is well accepted that at the same age, the variability in weight and organic development is very low (as observed in the present study in

CON mice). In addition, mice from the two groups were randomly divided at the beginning of the protocol to overcome this issue. The second limitation is the use of 1.5–2% isoflurane during MRI. Kober *et al.*<sup>46</sup> showed that with isoflurane levels between 1.25% and 2%, myocardial blood flow changes may modulate the heart hemodynamics. For MRI acquisition considerations (i.e. 15–17 frames per cardiac cycle), we chose to adjust isoflurane concentration to maintain comparable heart rate between mice. As they were inbred mice, we hypothesized that they need approximately the same isoflurane concentration. Indeed, there was no difference between groups in isoflurane concentration to maintain stable heart rate during acquisition. However, as the heart rate was artificially maintained by isoflurane, this measurement could not be used as a physiological parameter enabling to calculate cardiac output.

In conclusion, we have demonstrated that *in vivo* MRI results are related to *ex vivo* anatomical and biochemical adaptations to exercise training. As a result, MRI seems to be a valid methodology that could allow researchers to both reduce the number of animals required, and to perform a longitudinal follow-up of the heart hypertrophy/training level coupled with other organic characteristics by MRI.

**Author contributions:** All authors participated in interpretation of the results and review of the manuscript. EC-S, CM and HA participated in the study design; AB, MS, EA and FS conducted the MRI experiment; GDS, EA and EC conducted the postmortem experiments; and EA, CM, EC-S and HA wrote the manuscript. CM and EC-S equally contributed to the supervision of this work.

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## IX.2 Supplemental Research Data (not published)

### IX.2a BMDM differentiation

***In vitro macrophage differentiation.*** In order to assess the expression of M1 or M2 macrophages, we collected BMDM from C57/Bl6 mice and stimulated them with either IFN- $\gamma$  (induction of M1) or IL-4 (induction of M2) to verify the proper pro-inflammatory or anti-inflammatory cytokine response. As expected we found that anti-inflammatory cytokines are more expressed when stimulated with IL-4 and pro-inflammatory cytokines are more expressed when stimulated with IFN- $\gamma$  (See Figure 25). The next step was to determine whether oxidative stress had a role in the inflammatory pathways. We added a mimetic of anti-oxidant SOD (TEMPOL) or H<sub>2</sub>O<sub>2</sub> to the previous protocol to see whether the inductions would change. We found that TEMPOL was able to reduce the IFN- $\gamma$ -induced pro-inflammatory response while H<sub>2</sub>O<sub>2</sub> by itself did not further induce pro-inflammatory cytokines, but in combination with IFN $\gamma$  was able to increase expression of TNFa. In terms of anti-inflammatory cytokine induction, H<sub>2</sub>O<sub>2</sub> alone and in combination with IFN- $\gamma$  significantly reduced cytokine production.

From these experiments we can conclude that IFN- $\gamma$  induces M1 secreted cytokines and is further stimulated by oxidative stress and inhibited by anti-oxidants. IL-4 induces M2 secreted cytokines and is inhibited by oxidative stress. H<sub>2</sub>O<sub>2</sub> by itself did not seem to be as sufficient to induce a pro-inflammatory response as in combination with IFN- $\gamma$ . As low doses of oxidative stress can be beneficial, thus this result is not surprising.

Following these experiments, we sought to determine whether exercise training could have the same response as the oxidative stress/ anti-oxidant mimetics on macrophage



differentiation. However, because of a technical error, we were unable to use the ApoE<sup>-/-</sup> mice BMDM. Judging from the previous experiment results, we could expect that exercise training would induce the production of M2 cytokines.

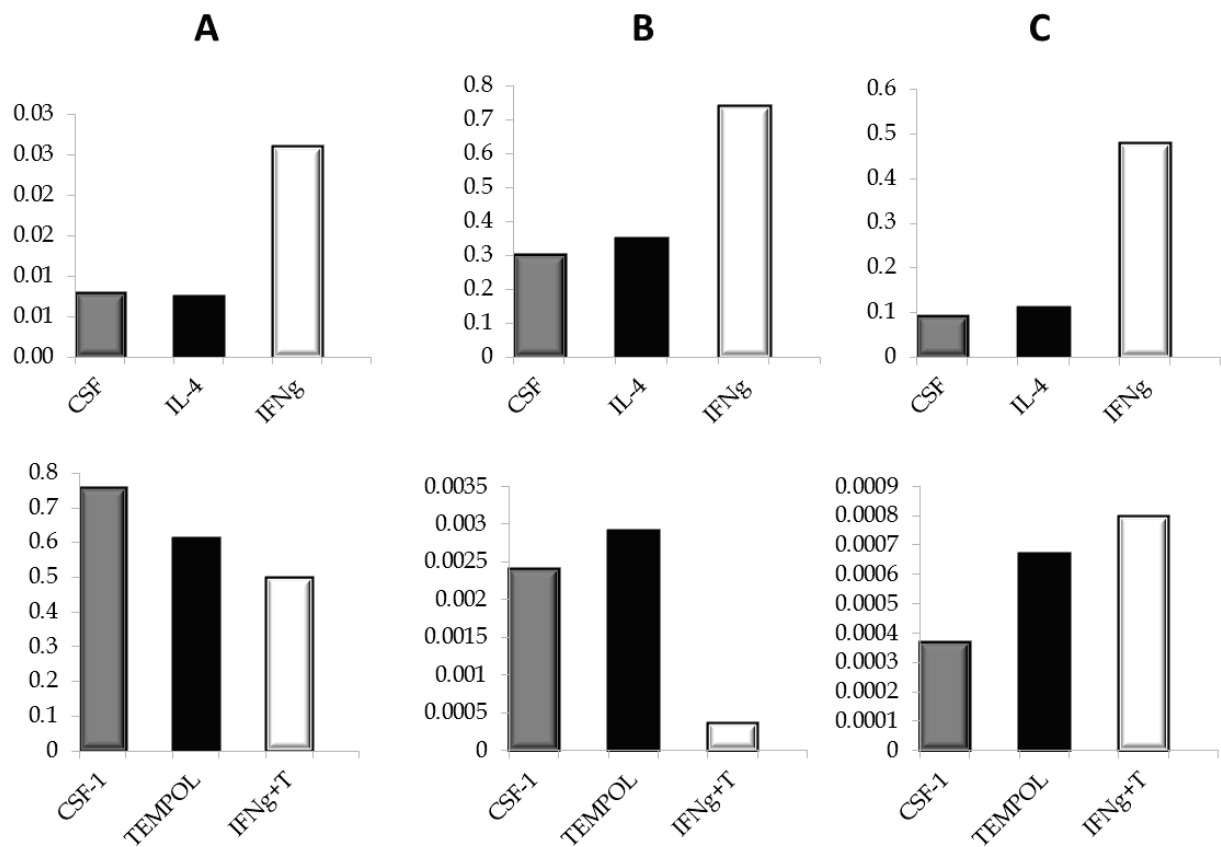


Figure 23: Differentiation of C57 BMDM.(A) TNF $\alpha$ , (B) iNOS, (C) IL-1 $\beta$ . Top row: Effects of M1 (IFN $\gamma$ ) or M2 (IL-4) stimulus on differentiation; Bottom row: Effect of anti-oxidant TEMPOL on M1 differentiation. CSF=colony stimulating factor-1; IL-4= interleukin-4; I