Magnetic Resonance Imaging Detected Intraplaque Haemorrhage as an Endogenous Imaging Biomarker and Therapeutic Target

by

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Abstract

Cardiovascular diseases, such as stroke and heart attack, are one of the largest causes of death and morbidity in Canada. Atherosclerosis, or the thickening of the arterial wall, has been identified as the primary culprit lesion behind the end organ damage associated with cardiovascular diseases. Magnetic resonance imaging has taken a primary role in characterising the constituents of these atherosclerotic plaques. Of these components, MR detected intraplaque haemorrhage (IPH), or bleeding inside the vessel wall, appears to predispose a patient to future clinical events. This leads us to the conclusion that IPH is a secondary manifestation of plaque progression and complication, or somehow contributes to the complication of these atherosclerotic plaques. This thesis explores this second possibility, in line with the “iron hypothesis” of
atherosclerosis which suggests that iron plays a significant, primary role in atherogenesis.

In chapter two, the signal hyperintensity associated with IPH is correlated with the lipid oxidising potential of blood products in various oxidation states. It is found that the ferric paramagnetic species has a significantly greater ability to generate lipid oxide components and oxidise lipid surrogates. This finding suggests that it may be possible to alter the course of plaque progression by inhibiting the active ferric iron state. Chapter three explores an endogenous molecule, haptoglobin, whose purpose is to bind and inactivate free haemoglobin. It is shown that haptoglobin has the ability to modulate the MR signal intensity from IPH. Chapter four explores a mechanism to detect this imaging biomarker outside the MR environment using the electron paramagnetic resonance of the ferric haemoglobin. Results are shown in a custom made bench top system detecting ferric haemoglobin in an in vitro sample.

This thesis provides more evidence for the iron hypothesis of atherosclerosis and explores methods of inhibiting and detecting this biomarker of disease.
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List of Abbreviations

AHA - American Heart Association

B_{eff} - Effective magnetic field

DEA-NONOate - Diethylamine NONOate, nitric oxide donor

DFO - Desferrioxamine

dHb - Deoxyhaemoglobin

EPR - Electron Paramagnetic Resonance

Fe^{2+} - Ferrous Iron

Fe^{3+} - Ferric Iron

H&E - Haematoxylin & Eosin

H_{2}O_{2} - Hydrogen peroxides

Hb - Haemoglobin

Hp - Haptoglobin

LDL - Low density lipoprotein

M - Net Magnetization

MDA - Malondialdehyde, a by product of lipid oxidation

mHb - Methaemoglobin

MRI - Magnetic Resonance Imaging

M_{xy} - Magnetization components perpendicular to the main magnetic field
M$_2$ - Magnetization components parallel to the main magnetic field

NO - nitric oxide

O$_2^*$ - Superoxide

OH$^*$ - Hydroxyl radical

oHb – Oxyhaemoglobin

PnA - Parinaric acid, fluorescent probe that quenches upon oxidation

RBC - Red Blood Cells

ROS - Reactive Oxygen Species

TI - Inversion Time

γ - Gyromagnetic Ratio

τ$_c$ - Correlation Time
Chapter 1: Overview of Thesis

1.1 Introduction

Cardiovascular diseases, such as stroke and heart attack, remain one of the largest causes of death and morbidity in Canada\(^1\). Statistics are similar in the rest of the western world, with these diseases accounting for over 30% of deaths in the United States of America\(^2\), and the United Kingdom\(^3\). However, these diseases affect not only first world countries; increasingly, rates of cardiovascular diseases in developing countries such as India and China are reaching levels similar to those found in North America\(^4\). Thus, cardiovascular disease has the potential to be an alarmingly large cause of mortality and morbidity worldwide.

Atherosclerosis, the thickening of the arterial wall, has been identified as the primary culprit lesion behind the end organ damage associated with cardiovascular diseases. When severe, this thickening encroaches into the blood carrying portion of the blood vessel, the lumen. Once blood flow is interrupted, downstream tissues are starved of much needed oxygen and nutrients that the blood supplies. This view of luminal encroachment leading to eventual occlusion of blood flow has been slowly evolving. We have started to recognize the blood vessel wall as a responsive organ rather than simply an inanimate conduit. It appears to be the disease within the blood vessel wall that contributes to the progression and clinical complication of atherosclerosis.

The following sections of this thesis outline the physiology of the normal artery wall and briefly describe one of the current views of plaque development and progression. We review some previous research aimed at characterising atherosclerotic plaque, with particular attention paid to intraplaque haemorrhage, a hallmark of advanced plaques. This thesis furthers the argument that plaque haemorrhage provides the necessary and sufficient fuel to drive future plaque progression. Clinical methods of detecting plaque haemorrhage are covered briefly, followed by an overview of the most recent results from population studies derived from
imaging data. Finally, a summary of Magnetic Resonance Imaging (MRI) is given that highlights the topics discussed in this thesis.

1.2 The Healthy Vessel Wall

In arteries that are over several millimetres in diameter, the vessel wall is made of three distinct layers: the intima, media and adventitia as shown in Figure 1.1.

![Figure 1.1 Relatively Healthy Vessel Wall](image)

The layers of the vessel wall are clearly visible in this Haematoxylin & Eosin (H&E) stained cross section of a rabbit vessel. The * denotes the luminal side, arrow indicating the single cell layer of endothelial cells. Early intimal thickening can be seen above the smooth muscle cell layer of the media, a dark pink band stained on this H&E slide. The adventitia, a region defined outside the external elastic lamina, is loose connective tissue often widely distributed with small blood vessels that supply nutrients to the smooth muscle cells of the vessel wall.

The intimal layer, or the layer closest to the blood carrying lumen, is lined by a monolayer of endothelial cells. This endothelial layer plays an important role in vessel wall health. It acts as an interface between the vessel and the flowing blood in the
lumen. This interface serves many purposes in maintaining vessel wall homeostasis including vasodilation and constriction, prevention of the ingress of unwanted proteins into the vascular wall, as well as regulation of blood clotting and thrombosis. As will be discussed in the following section, abnormality with normal endothelial function is the first step in vessel wall disease.

The adjacent medial layer is distinguished by a collection of smooth muscle cells that enable the vessel to constrict or dilate, regulating blood flow and blood pressure. This muscular layer is minimal in both the very large (e.g. aorta) and the very small (e.g. arterioles) arteries. However, germane to this thesis, the arteries that cause downstream symptoms (e.g. coronary and carotid arteries) all have a muscular layer that is responsible for the modulation of vascular tone.

The outer adventitial layer is largely considered extraneous to the artery itself. However, through this layer course the small arteries feeding the outer layers of the muscular media. These vessels, called the vasa vasora, (Latin for vessel of vessels) are critical in supplying blood flow to large arteries where the muscular layer is thicker than the diffusion distance of oxygen. These blood vessels are a conduit through which nutrients and oxygen are exchanged. Without these small vessels providing oxygen to the medial layers, an hypoxic region would result. As will be discussed below, the vasa vasora respond to the hypoxic condition by proliferating.

1.3 Plaque Development and Progression

One of the earliest markers of atherosclerotic disease is endothelial dysfunction, which predates any formation of pathologically visible plaque. This dysfunctional endothelial layer has a markedly reduced ability to mediate vasomotion, as well as a decreased ability to shield the vessel wall from unwanted particles in the circulation. One molecule in particular that merits more attention is the so called bad cholesterol, or low density lipoprotein (LDL). This macromolecule has been implicated as a crucial player in initiating the atherosclerotic process; increase in circulating levels of LDL is positively
correlated with incidences of cardiovascular diseases\textsuperscript{6}. Native LDL itself passes through the vessel wall continually, however ingress and modification of this protein is one of the first signs of the atherosclerotic process. It has been identified that oxidative modification of these LDL proteins acts as a powerful attractant to circulating monocytes\textsuperscript{7}. These monocytes burrow through the vessel wall, evolving into macrophages to engulf these foreign bodies. Once in the vessel wall and having gorged on the embedded LDL, the macrophage becomes trapped within the vessel wall and apoptoses. This is the earliest sign pathologists see on analysing the arteries post mortem - the fatty streak of macrophages filled with oxidised LDL\textsuperscript{8}.

While in the vessel wall, the macrophages release a host of inflammatory chemokines that encourage the smooth muscle cells within the media to migrate towards the luminal surface. In a process similar to scarring during the wound healing process, the vessel wall begins to thicken, the beginning of the atherosclerotic plaque.

![Progressive eccentric intimal hyperplasia with development of atherosclerosis in human arteries.](image)

**Figure 1.2:** Beginning of atherosclerotic plaque formation.

From left to right, a panel of H&E stained slides of increasing levels of atherosclerosis in human arteries. Eccentric thickening is seen from the left panel to the middle panel, with thickening predominantly from 3 to 5 o’clock position. However, it is worthwhile to note that the blood carrying lumen maintains approximately the same area to maintain blood flow in this intermediate disease state. Only when there is significant vessel wall disease (right panel) does the vessel wall encroachment begin to narrow the lumen.
The process of LDL infiltration followed by this wound healing process repeats itself over a lifetime, as illustrated in Figure 1.2. This type of plaque is prevalent in the majority of individuals over the age of 20 in western society; in a post mortem study over 60% of 20 year olds have abnormal thickening of their blood vessels\(^9\). However, plaque growth resulting in complete occlusion of blood flow is seen in only 15% of post mortem studies\(^10\); non-occlusive narrowing that causes thrombosis is actually a primary cause of death in the majority of cases of cardiovascular disease. This evidence suggests that there may be some trigger that switches a plaque from one that progresses slowly to one that makes an environment ripe for accelerated plaque progression that eventually leads to clinical symptoms. The search for causative mechanisms behind the “vulnerable plaque” has been a long standing research problem and this thesis seeks to add evidence towards a particular marker thought to make a plaque vulnerable.

The wound repair response that leads to the thickening of the medial layer may be one element that is responsible for this phenotypic switch from a stable to a fast growing plaque. The demand for oxygen increases once the medial layer becomes thicker than several cell layers. The perivascular vasa vasorum proliferates to meet this increased demand. This response is reminiscent of tumour hypoxic response in which neovascuclature is summoned from the surrounding tissues to supply the growing tumour with blood and nutrients. Unsurprisingly, the intraplaque neovasculature that forms in response to the hypoxic insult exhibits similar properties to the tumour neovasculature: it is tortuous, relatively unstructured and there is a low deposition of pericytes making the vasculature more permeable\(^11\). Increased permeability may lead to an increased extravasation of red blood cells. This permeability can be exacerbated in patients with hypertension, increasing the pressure on these fragile neovessels possibly causing them to rupture and leak red blood cells. This feature, denoted intraplaque haemorrhage (IPH), is a hallmark of “advanced” plaques as classified by the AHA\(^12\). This feature of an advanced atherosclerotic plaque is increasingly being recognised by many groups around the world as a possible driver of plaque progression, rather than simply a secondary outcome of endothelial dysfunction.
1.3.1 Intraplaque Haemorrhage

The new proliferative vasa vasora delivers red blood cells to the core of the plaque, ostensibly to deliver nutrients and oxygen. However, due to the increased permeability of the endothelial layer, some red cells extravasate and stay in the plaque. This local deposition of red blood cells, called intraplaque haemorrhage, appears to be a common component in advanced lesions. An example of such a lesion is seen in Figure 1.3.

It has been hypothesized by several groups that intraplaque haemorrhage is not only a component present in advanced lesions, but also one that is responsible for driving lesion advancement. One reason that is often cited is the rich cholesterol deposits that a red blood cell is capable of delivering to the body of the plaque. The red blood cell membrane has more free cholesterol than any other cell in the body\textsuperscript{13}, and recent evidence suggests that cholesterol found in plaques is predominantly red blood cell derived\textsuperscript{14}. These membrane constituents are delivered directly to the plaque core once the red blood cell lyses, several days after extravasation\textsuperscript{15}.

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Figure 1.3 Intraplaque Haemorrhage

An H&E stained cross section of a coronary artery obtained from the heart of a patient who died of acute myocardial infarction. The tightly stenosed lumen (*) can be seen with the fibrous cap (f) broken at the arrowhead. Intraplaque haemorrhage (+) is seen under this cap, with a surrounding necrotic core.
Furthermore, after red cell lysis, the contents of the cell are freed to interact with the environment. The predominant molecule inside these red cells is haemoglobin (Hb). Of particular interest in this molecule is the biologically active component, the iron in the middle of the haeme molecule. With over 70% of the body's iron stores in circulating Hb molecules, the red blood cell is able to deliver this metabolically active iron to the core of the plaque. While iron is critically important in its role of reversibly binding oxygen, once oxidatively modified, it has been shown to mediate a host of negative effects on vessel wall health. These effects are attributed primarily to the oxidative stress that ferric iron has on the surrounding vessel wall. Mediators of oxidative stress and effects will be outlined in more detail in the following sections.

1.3.2 Oxidative Stress
The idea of increased oxidative stress as the root cause behind aging\textsuperscript{16}, cancer\textsuperscript{17} and a host of other central nervous system ailments\textsuperscript{18} has been of increasing research interest in recent decades.

The most potent source of biological oxidization is a class of molecules collectively called the reactive oxygen species (ROS). In healthy tissues, ROS are produced as a consequence of aerobic respiration. Additionally, some ROS, such as nitric oxide (NO), are important intracellular signalling molecules mediating a wide array of vascular functions. The biological toxicity of these ROS must be kept in balance by mechanisms that re-oxidize the substrate of the ROS or bind and eliminate the ROS for degradation. This delicate balance between production and elimination can be significantly disturbed during disease.

Superoxide (O$_{2}^{•-}$), one of the most commonly found ROS, does not have a large potential for oxidizing other molecules owing to its short half life, existing only during its diffusion over several molecular radii. However, it is the interaction of O$_{2}^{•-}$ with water that produces the much longer lived hydrogen peroxide (H$_{2}$O$_{2}$). H$_{2}$O$_{2}$ can diffuse through mitochondria and cross cell membranes, thus bringing the ROS from its production site
in the mitochondria extracellularly. Like the superoxides from which it is formed, \( \text{H}_2\text{O}_2 \) has a low reactivity on its own. However, when in the presence of a transition metal (e.g. iron, copper or nickel) it has the potential to produce the most damaging ROS in mammalian cells, the hydroxyl radical (OH•).

Hydroxyl radicals have highly unstable electron structures and react with nearly any organic component in the near vicinity of their creation\(^{19}\). The reaction of transition metals and \( \text{H}_2\text{O}_2 \) is the well characterized Fenton reaction and is a widely used industrial process to eliminate organic wastes\(^{20}\). The transition metal catalyzes the formation of hydroxyl radicals and is itself reformed in the process, awaiting the catalysis of more peroxide molecules

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH} \cdot + \text{OH} \\
\text{Fe}^{3+} \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{2+} + \text{OOH} \cdot + \text{H}^+ 
\]

Thus, even minute quantities of both \( \text{H}_2\text{O}_2 \) and transition metals can create a potent oxidizing environment, one that needs very strict regulation.

The most abundant transition metal in the human body is iron. As discussed briefly in the previous section, the red blood cell is one of the body's largest repositories of iron, bound predominantly in the haeme molecule. During normal function, haeme is bound to globin (haemoglobin) and encased inside the erythrocyte membrane. However, during haemolysis, the membrane shell is compromised and haeme is allowed to leak extracellularly. Once outside of its protective casing, free haemoglobin is a potent scavenger of ROS. One such interaction shows that free haemoglobin rapidly reacts with NO; during this process haemoglobin is oxidized to methaemoglobin, changing the ferrous iron (Fe\(^{2+}\)) to its ferric form (Fe\(^{3+}\))\(^{21}\). Methaemoglobin has been shown to be a potent activator of endothelial cells\(^{22}\); it has also been shown to be a potent mediator of LDL oxidization\(^{23}\). The latter is mediated almost exclusively through the addition of \( \text{H}_2\text{O}_2 \). This evidence suggests that red blood cells (RBCs) have nearly all the requisite components to cause an inflammatory response intravascularly. Furthermore, it has been shown that RBCs, when forcibly inserted into an otherwise
quiescent vessel wall, have the potential to locally upregulate the number of macrophages in an animal model\textsuperscript{24}.

This evidence suggests that the red blood cell, in and of itself, is sufficient to cause increased inflammation and drive macrophage infiltration, which in turn promotes atherosclerosis. This cholesterol, while giving the red blood cell its rigidity and function, can act as the necessary substrate to cause an increased inflammatory response.

While the research discussed above hypothesizes a method of red blood cell mediated plaque formation (via endothelial injury) and progression (via oxidization of LDL intravascularly), the large flow velocities in the intravascular environment allow very little time for the interaction of even lysed red blood cells with the vascular wall. Additionally, as will be detailed further, the body’s endogenous defense mechanisms against red blood cell mediated damage is formidable in an intravascular compartment. Thus, to truly drive the progression of the atherosclerotic process, these reactions must occur intramurally.

1.3.3 Summary of Plaque Progression Hypothesis

The picture painted thus far looks as follows: following endothelial dysfunction, an accumulation of oxidatively modified LDL attracts monocytes into the arterial wall. These monocytes differentiate into macrophages that engulf the oxidised LDL, stimulating an inflammatory process. The inflammation drives the medial muscular layer to migrate and proliferate, generating a thickened medial layer. Once the vessel wall is sufficiently damaged, the vasa vasorum proliferates to feed the hypoxic core in the thickened media. Due to the permeable nature of this vasculature, red blood cells leak out and remain at the plaque core, delivering both a rich source of unoxidized cholesterol in their membranes as well as a powerful oxidizing agent in the form of metabolically active iron.

This intraplaque haemorrhage may be the switch that accelerates plaque progression. Once the plaque is infiltrated by red blood cells, a nidus of inflammatory...
Effects is deposited in the core and the plaque progression process may be many times accelerated. LDL enters the highly oxidative environment of the plaque core containing iron rich intraplaque haemorrhage. This LDL oxidizes and the plaque progression cycle repeats whereby this oxidized LDL attracts more monocytes, which in turn create more inflammation, driving hypoxia, and recruiting more neovessels, leading again to plaque haemorrhage. If these hypotheses are correct, the phenotypic switch that accelerates plaque growth may be the ingress of free haemoglobin, with its high potential to generate ROS, quickly spiraling into further plaque growth. Being able to reverse this switch may be a crucial component in slowing down the progression of atherosclerotic plaque.

1.4 Non Invasive Detection of Intraplaque Haemorrhage

Early attempts to non-invasively identify intraplaque haemorrhage focused primarily on using duplex ultrasound. These early studies concluded that haemorrhage could not be reliably detected because recent haemorrhage gave hypoechoic signal whereas older haemorrhage gave hyperechoic signals that mimic calcification\textsuperscript{25,26}.

In 2001, Yuan et al. demonstrated that magnetic resonance imaging (MRI) was capable of characterizing components of plaque\textsuperscript{27}. Later Moody et al. showed that intraplaque haemorrhage in the carotid arteries can be detected using MRI\textsuperscript{28}. This study used a three dimensional gradient echo sequence, suppressing signal from other species that are hyper intense in a T\textsubscript{1} weighted imaging sequence. Patients presenting with cerebral ischemia had a higher prevalence of signal hyperintensity in the carotid arteries than those presenting without cerebral ischemia as shown in Figure 1.4.
Others have been quick to adopt this sequence in the monitoring of patients presenting with cerebral ischemic symptoms. These studies have generated population outcomes with results that support the “intraplaque haemorrhage as a phenotypic switch”

Figure 1.4 MR Detected Intraplaque Haemorrhage Identifies Patients with Increased Risk of Cerebrovascular Events Independent of Stenosis

Panel A shows a coronal slice of a T1 weighted three dimensional, spoiled gradient echo volume that is fat and flow suppressed. Focal T1 hyperintensity, denoted by the arrowhead, appearing in the left carotid bifurcation is indicative of intraplaque haemorrhage in the arterial wall. Furthermore, natural history trials have correlated this presentation to an increased risk of future clinical events. Inset is a bright blood MR angiogram showing stenosis at the carotid bifurcation of the same artery. This level of stenosis is similar to that of the stenosis seen in the inset of panel B. However, despite having similar stenosis, the absence of a T1 hypertintense signal is suggestive of stable plaque morphology.
hypothesis. Takaya et. al. have shown that of the various imaging characteristics found on MRI, the only finding that was predictive of plaque growth was intraplaque haemorrhage. Altaf followed a cohort of symptomatic, high grade (>70%) stenosis patients and found that intraplaque haemorrhage positive MRI differentiated patients in terms of clinical outcome. MRI positive patients were found to have worse outcomes than patients that were MRI negative. Likewise, this same group found similar results in moderate grade, again with MRI positivity showing negative patient results. Similarly, Singh et. al. have shown a very similar outcome, this time in patients who were asymptomatic that had mid grade stenosis. To further elaborate on the research discussed within this thesis, a brief overview of MRI will be given that addresses issues germane to this work. For a detailed explanation of MRI, refer to one of the many excellent texts that cover this topic more completely.

1.5 Brief Overview of MRI

Nuclear Magnetic Resonance is a phenomenon exhibited when nuclei in a magnetic field absorb energy from an applied radio frequency pulse. This absorbed energy causes the spin state of the nuclei to flip into a high energy state. While this phenomenon is a manifestation of quantum electrodynamics, a very large ensemble of these spins can be accurately described using a classical description. This chapter will concentrate on the classical description of MR as it accurately represents the features of MRI most of interest to this thesis.

The phenomenological equation that describes the classical behaviour of a system of spins, known now as the Bloch equation is:

$$\frac{dM}{dt} = \gamma M \times B + \frac{1}{T_1}(M_0 - M_z)\vec{z} - \frac{1}{T_2}\vec{x}\vec{y}$$  

(3)

This differential equation dictates much of the behaviour of MR and contains a wealth of information that governs the behaviour of samples in a magnetic field.
The first term in the Bloch equation describes the evolution of the net magnetization, \( M \). It is useful to divide \( M \) into components parallel to the main magnetic B field, \( M_z \), as well as components perpendicular to the B field, \( M_{xy} \). If we ignore relaxation, the change of the \( M_z \) as a function of time is zero, and, the perpendicular components change proportional to the cross product of \( M \) and a magnetic field, \( B \). This B field is often referred to as the effective magnetic field, denoted \( B_{\text{eff}} \), which includes not only the static main magnetic field, but also any externally applied sources. In the absence of any applied B field, the \( B_{\text{eff}} \) is along the main magnetic field and any \( M_{xy} \) will rotate, or precess, around the longitudinal axis. The gyromagnetic ratio \( \gamma \) is a proportionality constant that is determined for each element. Of interest to this thesis is the hydrogen nucleus which has a \( \gamma \) of approximately 42.57 MHz / Tesla.

The next set of terms describes the change in magnetization as a function of two distinct relaxation mechanisms. The second term in the Bloch equations is an additive regrowth term that restores the magnetization to the original net magnetization, \( M_0 \). This regrowth is dependent on the size difference between magnetization oriented along the \( z \) axis, and \( M_0 \), as well as a rate constant term, \( T_1 \). The final term, which we mention here for completeness is a term that represents the decay of signal with respect to time. While this term has very important effects and consequences in MRI, images can be acquired that rely heavily on the first regrowth term, and thus are able to largely ignore \( T_2 \) relaxation. This thesis focuses more on the first relaxation mechanism, \( T_1 \), which is discussed in further detail in the following section.

1.5.1 Excitation and Regrowth of Net Magnetization

Without the addition of energy into the system, \( M \) eventually orients along \( B \). To detect a signal from this magnetization by induction, a time dependent change is required to induce a voltage in a nearby receiver coil. This criterion is not met by the longitudinally oriented magnetization; to change the orientation of this magnetization, a secondary B field, perpendicular to the main magnetic field needs to be applied. This field, in resonance with the rotating magnetization, will rotate \( M \) away from the \( z \) axis.
Ignoring the first term of the Bloch equation, the parallel component $M_z$ has a solution that describes an exponential regrowth towards $M_0$.

$$M_z(t) = M_z(0)e^{-t/T_1} + M_0(1 - e^{-t/T_1})$$ (4)

The regrowth of longitudinal magnetization is the result of a loss of energy from the excited spin system into the surrounding environment. As in the excitation of the spin system, this exchange of energy only happens at the resonant frequency. Thus, this relaxation process is dependent on a magnetic field that fluctuates in the environment at the resonant frequency. Such fluctuations are brought about by the Brownian molecular motion of the molecules surrounding the excited spin. Again, it is important to note that without these magnetic field fluctuations, the relaxation of an excited spin happens exceedingly slowly.

Brownian motion is affected by the size and the conformation of molecules. **Correlation time**, often denoted $\tau_c$, describes the average time it takes a molecule to rotate by one radian. This metric is useful to understand the relative mobility of the molecules in question. Very large macromolecular proteins, such as those anchored in membranes, tumble slowly and thus have a long $\tau_c$, or inversely, a low tumbling frequency. These make poor reservoirs into which energy can be exchanged. The rate of relaxation of an excited spin in this system is quite slow and correspondingly, the $T_1$ of these substances is quite long. Similarly, very small molecules, such as water, reorient themselves quickly and cause fluctuations much higher than the Larmor frequency. These molecules have a very small $\tau_c$, or inversely a very high tumbling frequency. These too make a poor reservoir to which spins can lose their energy. Both of these scenarios represent situations in which relaxation to thermal equilibrium is slow, representative of a long $T_1$. At magnetic field strengths commonly used clinically today, this relaxation is on the order of seconds.

Triglyceride type molecules with long, free rotating hydrocarbon chains where the bulk tumbling rate of these molecules approximates the Larmor frequency facilitates energy exchange. These molecules return the excited spin states to thermal equilibrium.
quickly, resulting in a short \( T_1 \). Again, at clinical field strengths, the \( T_1 \) of these fatty molecules is on the order of a few hundred milliseconds.

### 1.5.2 Paramagnetic Material and Relaxation

The scenario regarding energy transferred to a nearby tumbling molecule as described in the preceding section also holds true when it comes to the relaxation caused by paramagnetic substances. However, in this case the absorption of energy is not due to a neighbouring nucleus that is tumbling at the resonant frequency, but rather an unpaired electron. Similar to the nucleus, the electron has a magnetic moment; however this moment is nearly seven hundred times the size of the nuclear magnetic moment. Thus, even though the \( \tau_c \) of these electrons is significantly shorter than the resonant tumbling rate, the increased magnetic moment more than offsets the rapid tumbling of these electrons. These unpaired electrons have a dramatic effect on the nuclear relaxation. This is used extensively in the design of MR contrast agents that use elements with large numbers of unpaired electrons to generate a local enhancement in relaxivity. Most typically used is some form of chelated Gadolinium, an element that has five free electrons. Other paramagnetic materials have been used in the production of magnetic resonance contrast agents such as the rare earth Europium, as well as other more commonplace elements such as iron.

### 1.5.3 \( T_1 \) Imaging

\( T_1 \) is an important relaxation parameter that is used to generate contrast between different tissues in MR imaging. To detect the differences between the tissues based on their \( T_1 \), data is acquired during the relaxation period that provides different signal intensity for the different tissue types. By applying a train of RF excitation pulses with a repetition time that is shorter than \( T_1 \), complete relaxation to \( M_0 \) will not occur. Rather, tissues with a short \( T_1 \) with respect to the repetition time will have more recovered magnetization, and thus higher signal intensity on the subsequent RF pulse compared
to tissues with very slow $T_1$ relaxation. Continuing to apply these pulses, the magnetization eventually reaches a steady state where short $T_1$ species will have higher signal intensity, whereas long $T_1$ species will have relatively lower signal intensity. By modulating the repetition time, it is possible to achieve a wide range of $T_1$ contrast. Using repetition times longer than the $T_1$ of all tissues in the imaging volume, say several seconds, generates an image that is relatively uniform in contrast, with the only difference being a change in signal intensity based on the density of the protons. However, as repetition times shorten, tissues with the longest $T_1$ will lose signal, accentuating those with very short $T_1$. As repetition times shorten to mere milliseconds, such as in sequences used to detect plaque haemorrhage, only those species with a very short $T_1$ will appear with a signal hyperintensity compared to the rest of the image, which will be of moderate to low signal intensity.

### 1.5.4 Measuring $T_1$

To quantify the $T_1$ of tissues, methods have been developed that trade off various factors, usually for faster acquisition time. The gold standard technique is an inversion recovery scheme where an initial burst of RF energy is used to invert the magnetization in the system. This inversion leaves the net magnetization oriented opposing the main magnetic field. This magnetization recovers towards $M_0$ according to:

$$M_2(t) = M_0 \left(1 - 2e^{-t/T_1}\right)$$

Following some delay, TI, the magnetization is excited into the perpendicular plane and the signal is recorded. By repeating this experiment and changing the TI, the recovery curve of the magnetization can be determined exactly. The down side of this method of determining $T_1$ is that the repetition time between subsequent inversions is very long – often more than five times $T_1$ to ensure significant relaxation to the equilibrium steady state. Thus repetition times of tens of seconds are not uncommon – this results in experimental measurements that last hours given enough sample points along the recovery curve, combined with enough samples to accurately construct an image.
An alternative to acquiring only one data point per inversion is using a scheme proposed by Look and Locker whereby the magnetization is sampled during the regrowth period. Here, instead of using the entire bulk of the magnetization in the \( M_z \) axis, a limited tip angle is used to nominally disturb the regrowth of the magnetization. The magnetization no longer recovers as \( T_1 \) because it is disturbed during the recovery process. However, \( T_1 \) may be determined using a three parameter fit to the equation

\[
S(t) = A - Be^{(-t/T_1^*)}
\]

where \( S(t) \) is our measured signal, \( T_1^* \) is the apparent relaxivity, \( A = M_0 \frac{T_1}{T_1} \) and \( B = M_0 (1 + \frac{T_1}{T_1}) \). From this fit data, we can solve for \( T_1 \) as

\[
T_1 = T_1^* \left( \frac{B}{A} - 1 \right)
\]

1.6 Chapter 1 Summary

The scientific community has shown that intraplaque haemorrhage detected in histological specimens appears to identify patients at risk for future systemic cardiovascular events. Furthermore, through the previous discussion, our work, and the work of others it appears that MR detected intraplaque haemorrhage is a predictor of future clinical events in both symptomatic and asymptomatic patient populations. What still remains to be shown however, is a clear association between the signal hyperintensity detected in MRI and evidence that this is associated with an environment that accelerates plaque progression. The hypothesis that this hyperintensity is associated with blood products which enhance lipid oxidation has not been demonstrated. Additionally, it is unknown whether this MR finding is more likely to be found in a sub group of patients that identify themselves via some other means such as gender or a genetic marker.

The remainder of this thesis further explores the biological effects of intraplaque haemorrhage as well as the presentation of IPH on MRI. Chapter 2 investigates the relationship between the MRI signal and the oxidation states of various blood products.
in vitro. We identify ferric haemoglobin as a potent oxidizer of low density lipoprotein lipid; furthermore, this blood product generates a signal hyperintensity when imaged under clinical $T_1$ weighted imaging. This evidence suggests that the underlying reason that patients present with signal hyperintensity at the carotid bulb is a local increase in lipid oxidation leading to further inflammation.

In Chapter 3, we show that the ferric iron blood product identified in chapter 2 can be modulated with endogenous and exogenous compounds. Furthermore, it may be possible to monitor their effect using conventional MRI.

Chapter 4 presents some initial steps towards a detection scheme that brings the patient outside of the clinical MRI environment into a situation where it may be possible to do low cost detection of problematic plaques.

Chapter 5 concludes with some works in progress and some ideas for the future, which may answer some of the questions posed in this thesis.
1.7 References for Chapter 1


Chapter 2 MRI Detects Oxidative Stress Induced by Methaemoglobin

This chapter is largely reproduced from a paper published in Radiology

2.1 Introduction

Magnetic resonance imaging is a useful tool for characterising atheroma, particularly in the carotid artery\(^1\). Multiparametric MRI is able to differentiate the plaque’s necrotic core from the fibrous cap, and identify significant features of plaque complication such as cap disruption, and intraplaque haemorrhage\(^2\). IPH produces a focal hyperintensity on T\(_1\) weighted MR images when signal from both fat and flowing blood are suppressed\(^3\). IPH appears to be prognostic for both plaque progression and patient outcome; in a small multiyear study, IPH uniquely identified patients who had plaque growth\(^4\). IPH also differentiated patients who were at increased risk of future stroke while awaiting endarterectomy\(^5\), and predicted future events in a patient population with >50\% carotid stenosis\(^6\). This finding held true even in asymptomatic patients with moderate stenosis\(^7\). Moreover, the data presented in these papers suggested that the absence of this imaging marker indicated increased plaque stability with significantly decreased risk of future ischaemic events.

This evidence suggests that MRI can be used as a tool to identify patients at risk for future clinical events using IPH as a surrogate marker. In this setting, IPH may simply represent a secondary manifestation of plaque progression or complication. Alternatively, the “iron hypothesis” of atherosclerosis\(^8\) theorises that iron plays a significant primary role in atherogenesis. Red blood cells (RBCs) within IPH may contribute to increased plaque progression through two mechanisms. Firstly, haemoglobin (Hb) within the red blood cell contains haeme and molecular iron, the latter being a potent generator of free radicals\(^9\). These free radicals are capable of potently
oxidising low density lipoprotein (LDL). It is accepted that oxidative modification of LDL first causes then drives plaque development\(^1\). Secondly, the red blood cell membrane contains high concentrations of LDL\(^1\) that contribute to the plaque lipid core. Both of the components necessary to drive plaque progression, the lipid rich membrane and the inflammatory stimulus from molecular iron are therefore delivered within the plaque with each red blood cell during IPH.

The purpose of this chapter is to correlate the effect of red blood cell haemoglobin on MRI signal generation and low density lipoprotein oxidation.

### 2.2 Materials and Methods

#### 2.2.1 Sample Collection and Red Blood Cell Preparation

Informed consent was obtained from six volunteers participating in this study between the dates of June 2008 and June 2009 as approved by the research ethics board. Venipuncture of an antecubital vein was performed with a butterfly infusion set and whole blood was drawn into vials containing lithium heparin (BD Vacutainers). Oxygen saturation was increased by bubbling pure O\(_2\) gas to generate oxyhaemoglobin (oHb). Blood was deoxygenated by bubbling pure N\(_2\) gas forming deoxyhaemoglobin (dHb). Once formed, dHb was kept in an airtight container topped with N\(_2\) at atmospheric pressure and used within 2 hours of preparation. Ferric methaemoglobin (mHb) was prepared by incubating oHb blood specimens and the nitric oxide donor diethylamine-NONOate (Sigma D5431) for at least 20 minutes at room temperature. To verify that the inactive byproducts of DEA-NONOate did not affect our observations, the compound was warmed to room temperature for two days prior to addition to the haeme samples. The half life of DEA-NONOate is approximately 16 minutes at room temperature; after approximately 200 half lives, the NO release is negligible.

Red blood cells were isolated by centrifugation at 1250\(g\) for 10 minutes and the supernatant was discarded. These cells were then washed and resuspended in phosphate buffered saline and the process repeated three times. Extracellular RBCs
were prepared by placing them in a hypotonic solution of five volumes of double deionised water and vortexed at room temperature for 15 minutes. The slurry was then centrifuged at 1250g for 10 minutes and passed through a 0.22 μm filter to remove the membranes.

2.2.2 Product Quantitation

Hb concentration was determined by adding 20 μL of whole blood to Drabkin’s Reagent (Sigma-Aldrich D5941). This agent modifies the blood to a uniform type, cyanmethemoglobin, which has a characteristic absorption peak at approximately 530nm.

Stock solution of haemoglobin (Sigma-Aldrich H7379) was mixed with Drabkin’s Reagent to create a cyanmethaemoglobin standard curve with distribution of 0, 60, 120, and 180mg/mL. The absorption of these samples was measured at 530nm on a spectrophotometer (Beckman Coulter DU730). A straight line fit was used to obtain the relationship between absorption and haemoglobin concentration. Concentration of haemoglobin of samples in mg/mL was determined using the above determined fit. To convert to molar concentrations, a molar mass of 64.5 kilograms / mole was used.

Figure 2.1 Absorption Spectra of Different Oxidation States of Blood

Oxy (red curve) and Deoxy (blue curve) haemoglobin have characteristic absorption curves between 520 and 600nm. Met(black curve) haemoglobin does not. Absorption data obtained from Ziljstra et al.
To obtain mHb, oHb and dHb proportions in samples, absorption data was attained by recording a sweep across the spectrum from 390 nanometers (nm) to 600nm as shown in Figure 2.1 in 5nm increments using the same spectrophotometer. Using values published in the literature the absorption data was fit using a Nelder Mead simplex approach as implemented in Matlab, over the entire spectrum using the parameters

\[ A \cdot oHb_{spec} + B \cdot dHb_{spec} + C \cdot mHb_{spec} = measured_{spec} \]  

(8)

Where \( xHb_{spec} \) is a vector containing the literature values of optical absorption at each measured point for all the oxidation states. Percentages were calculated by dividing the free parameters by the sum of the total A, B and C. All blood samples were prepared within four hours of collection and assayed in duplicate.

2.2.3 Magnetic Resonance Measures of \( R_1 \)

All samples were measured in a 3T Philips Achieva system (Philips Healthcare, Best, Netherlands) where the specimens were placed in a transmit/receive quadrature head coil. The coil was loaded with a flat water phantom to ensure accurate RF power scaling. Samples were prepared in a 96 deep well plate with well volumes of 600μL. 50, 100, 150 and 200μL of blood solution were added to the wells yielding concentrations ranging from 619uM to 3.17mM determined as described above.

\[ R_1 = 1/T_1 \]

was estimated using a single slice, 2D Look-Locker technique to measure signal after inversion recovery. An adiabatic pulse was used to minimize the effects of \( B_1 \) inhomogeneity on the inversion pulse. The Look-Locker technique was chosen as the total acquisition time for a single slice was 59 seconds which was sufficiently quick to avoid confounding effects caused by the settling of intact RBCs. RBCs were agitated prior to scanning to resuspend any that had settled to the bottom of the wells. Pulse sequence parameters include TR/TE/\( \theta \) = 19ms/5ms/5°, slice thickness of 10mm, in plane resolution of 1x1mm over an 8 cm field of view. An EPI readout was used, acquiring 9 lines of k-space per excitation in centric view ordering. 192 points
were sampled along the recovery curve and a 3000 millisecond delay was placed after the final readout to allow regrowth of longitudinal magnetisation.

Total time between inversions was 6,648ms. To determine the longest $T_1$ for which this time would yield a recovery of 95% of $M_0$ we treat the magnetization from the first portion of the Look-Locker acquisition as equivalent to the steady state gradient echo signal:

$$M_{ss} = \frac{1 - e^{-TR/T_1}}{1 - e^{-TR/T_1}\cos(\theta)}$$

The second phase involves no RF excitation thus the signal grows exponentially towards $M_0$ over the 3000ms recovery time.

$$M_s(t) = M_{ss}e^{-t/T_1} + \left(1 - e^{-t/T_1}\right)M_0$$

Substituting appropriate values we see that with a small flip angle of approximately 5 degrees, and $T_1$ of 1500 ms, longer than we would expect to see, we have a steady state magnetization of over 75% $M_0$. After the three second delay, 97% of the original magnetization will have regrown.

Data were analysed in MATLAB where modulus mono exponential curves were fit using a Nelder-Mead simplex approach to determine the value of $1/(T_1^*) = R_1^*$ for each specimen. $R_1$ was then calculated on a voxel by voxel basis using a standard method of correcting for signal attenuation following a Look-Locker acquisition as detailed in the introduction chapter. The mean value of each sample ROI was taken as the $R_1$ and the standard deviation was calculated from the ensemble of pixels.

The relaxivity, $r_1$, of an $R_1$ enhancing agent in fast exchange is calculated as a straight line fit to the formula

$$R_{1\text{apparent}} = R_{1\text{actual}} + r_1[Agent]$$
where $R_{\text{apparent}}$ is $R_1$ measured from experiment, $R_{\text{actual}}$ is the $R_1$ of the substance in the absence of any contrast media, and $[\text{Agent}]$ is the concentration of the contrast media. A confidence interval for the straight line fit based on the standard deviation for all the pixels was calculated.

2.2.4 Measuring Lipid Peroxidation

To measure the presence of lipid peroxidation we assayed for thiobarbituric acid reactive species (TBARS) (Cayman Chemical 10009055), a sensitive but nonspecific test for the presence of malondialdehyde (MDA), a known by product of lipid peroxidation. Samples were assayed in duplicate as previously described\textsuperscript{14}. Briefly, 100μL of sample was incubated with 100μL of sodium dodecyl sulfate and 50pg of purified LDL (Sigma L7914). This solution was then boiled with thiobarbituric acid for one hour. Samples were then centrifuged at 1600g at 4 degrees Celsius for 20 minutes and the clear supernatant was spectrophotometrically assayed reading the absorbance at 530nm on a plate reader (Power Wave X340, Biotek Instruments). The absorbance was converted to a concentration through the use of a standard curve where known concentrations of MDA (0, 20 and increasing by 20 to 100μM) were assayed also at 530nm. A straight line fit was made through this absorption data and the equation of this line was used to calculated the concentration of MDA.

A second less destructive test of lipid peroxidation was performed using the LDL analog cis-parinaric acid (PnA) (Invitrogen P36005). Other groups have used the compounds dichlorofluorescine and dichlororhodamine to assay similar properties. However, these agents are known to rapidly oxidise in the presence of haeme products and are not indicative of lipid oxidation\textsuperscript{15}. Amongst the currently available fluorescent probes, PnA is the closest structural analog to endogenous membrane lipoproteins\textsuperscript{16}. PnA fluoresces at 420nm when excited by UV light. Fluorescence activity is lost when the chromophore is cleaved during oxidation of the PnA particle. PnA was allowed to incorporate with LDL by adding an aliquot of an ethanolic solution containing 1.5nM of PnA to 2 ml of buffer containing 30 pg of LDL (Sigma L7914). The incorporation was allowed to proceed for 5 min with gentle stirring. This solution was then added to each
test vial instead of pure LDL protein. Each sample was loaded into a clear cuvette and a 305nm laser was used to excite the contents of the cuvette. The beam path was then filtered using a 400nm long pass filter (Thorlabs FGL400) and emission data were summed every 2nm between 410 and 460nm.

2.2.5 Statistical Tests

Two tailed Student’s t-tests were used to determine if there were significant differences between means. Pearson’s correlation coefficient was calculated to determine the linearity of the data fit. 95% confidence intervals were calculated from this linear fit.

2.3 Results

2.3.1 Blood Product Production

Blood product composition was spectrophotometrically measured and the proportion of each blood product calculated for all n=6 samples; mean and standard deviations are reported in Table 1. The composition of dHb is contaminated with non negligible quantities of oHb as any perturbation of the dHb inevitably draws some oxygen into solution. No significant differences in spectrophotometric composition were measured between blood that had been exposed to the degraded DEA-NONOate substance and oHb and dHb products that had not.
MRI Detects Oxidative Stress Induced by Methaemoglobin

<table>
<thead>
<tr>
<th></th>
<th>%Oxyhaemoglobin</th>
<th>%Deoxyhaemoglobin</th>
<th>%Methaemoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td>oHb</td>
<td>96.0 +/- 0.4</td>
<td>3.1 +/- 0.2</td>
<td>0.9 +/- 0.5</td>
</tr>
<tr>
<td>dHb</td>
<td>22.0 +/- 0.1</td>
<td>76.9 +/- 1.0</td>
<td>1.1 +/- 1.0</td>
</tr>
<tr>
<td>mHb</td>
<td>0 +/- 0.1</td>
<td>0.1 +/- 0.7</td>
<td>99.8 +/- 0.7</td>
</tr>
</tbody>
</table>

Table 1: Blood Product Production

The spectrophotometrically measured composition of each of the blood products is shown above. While the oHb and the mHb samples are predominantly oxyhaemoglobin and methaemoglobin respectively, non-negligible concentrations of oxyhaemoglobin are in the dHb sample as no inhibitor was added to prevent deoxyhaemoglobin from rebinding with oxygen.
2.3.2 MR Measured Relaxivity Show High Values for Lipid Oxidation Active Species

Figure 2.2 shows an example mono exponential fit for R1* determination as well as linear fit for r1 determination. Pearson’s R² value of greater than 0.9 was achieved for all fits.

![Graph](image)

Figure 2.2 Representative Data Showing Fit of Data to Models

Panel left shows representative data from one sample; the modulus exponential data obtained from the Look-Locker experiment (x) is plotted along with the fit line (solid line). The R₁* is obtained as a fit parameter and corrected as detailed in the text to generate an R₁ value. These corrected R₁ are plotted as a function of concentration (right panel) and a straight line is fit through these data points. The data in the right panel is for methaemoglobin with a straight line fit slope of 0.324 and a Pearson’s R² value of 0.9682.
Figure 2.3 depicts the calculated r1 values for intra and extracellular haeme products in their ferrous and ferric states. Error bars indicate 95% confidence intervals calculated from the linear regression. A small increase in the r1 value is seen for both dHb and extracellular dHb over the values of oHb and extracellular oHb; however, both intra and extracellular mHb have approximately a 10 times higher relaxivity than either oHb and dHb.

Figure 2.3 Ferric Hb has a Greater Relaxivity than Ferrous Hb

In its ferrous oxy and deoxy forms, haemoglobin is not a strong T1 relaxation agent, as shown by the low calculated r1 (concentration independent measure of relaxation) values. However, on conversion to its ferric met form, Hb generates significant T1 relaxation. No significant differences were found between the intra and the extracellular forms. oHb is oxyhaemoglobin, dHb deoxyhaemoglobin and mHb methaemoglobin. The e prefix indicates extracellular.
2.3.3 TBARS Increases After Addition of Haeme Products

To determine the oxidative stress generated by haeme products in their various oxidation states, LDL, and H₂O₂ were incubated and then assayed for reactive MDA using TBARS assay. As shown in Figure 2.4 no detectable amount of MDA production occurs when LDL is incubated alone, or in the presence of H₂O₂.

![Graph showing calculated MDA (μM) for different conditions](image)

Figure 2.4 Ferric Hb catalyses more MDA production

No appreciable amount of malondialdehyde (MDA), a byproduct of lipid oxidation, is produced in the absence of haemoglobin (Hb) products. However, after the addition of 1mM of Hb in various forms, there is a distinct and measurable increase in MDA from baseline (p<0.00001) indicating increased lipid oxidation. Additionally, there is greater MDA production after the addition of mHb than either oHb or dHb (p<0.001). This suggests that mHb is more easily able to oxidize LDL than either of the ferrous counterparts. Error bars are standard deviation of the mean from three separate trials. Here H₂O₂ is hydrogen peroxide, LDL is low density lipoprotein, oHb is oxyhaemoglobin, dHb deoxyhaemoglobin and mHb methaemoglobin.
However, these oxidation effects are significantly enhanced after the addition of 1 mM of oHb, dHb and mHb (p<0.00001). Greater MDA production is seen on the addition of mHb than either oHb or dHb (p<0.001).

2.3.4 PnA Measures Demonstrate a Difference Between Intracellular and Extracellular Ferric Hb

To differentiate between intracellular and extracellular mediated generation of oxidation, the less destructive PnA assay was used to measure surrogate lipid oxidation. Figure 2.5 shows the cumulative fluorescence of PnA when incubated with haeme products in intracellular and extracellular oxidation states. Small decreases in PnA fluorescence are seen after the addition of 1 mM concentration of oHb and dHb. These values change little upon lysis, however, intracellular ferric Hb shows a larger decrease in PnA fluorescence compared with the ferrous iron products and extracellular ferric Hb exerts a significant effect on PnA fluorescence.
Figure 2.5 Ferric Hb quenches more PnA Fluorescence than Ferrous Hb

In the absence of haemoglobin (Hb) products, no cis-parinaric Acid (PnA) fluorescence is lost. However, upon addition of ferrous Hb, there is an increasing amount of fluorescence lost. Substantial fluorescence quenching is seen on the addition of extracellular mHb, with significantly less seen when using an intracellular product. This suggests that the cellular membrane is an important inhibitor of lipid oxidation via Hb sequestration. Error bars are standard deviation of the mean from three separate trials. PnA is a mixture of LDL and cis-parinaric acid, H2O2 is hydrogen peroxide, X is the PnA, LDL and H2O2 solution, oHb is oxyhaemoglobin, dHb deoxyhaemoglobin and mHb methaemoglobin. The e prefix indicates extracellular.
2.4 Discussion

We have demonstrated a source of signal hyperintensity on T₁-weighted MRI, ferric haeme, which provides an environment that readily oxidises low density lipoproteins. This hyperintensity may provide an endogenous contrast agent reflecting vessel wall disease activity in the setting of intraplaque haemorrhage. Additionally, this finding may account for the proven association of vessel wall high T₁ signal intensity with plaque progression, poor patient outcome and future neuroischaemic events.

The MR measures here agree with work performed previously showing that ferric Hb products have a higher relaxivity compared to ferrous Hb products. The quantitative measures in this paper may potentially be useful in longitudinal studies of patients. In this scenario, quantitative measures of T₁ may yield information about the time varying concentrations of ferric Hb. Furthermore, micro haemorrhage into the atherosclerotic plaque may be identified by a focal change in relaxivity. This may improve the detection threshold for intraplaque haemorrhage from the current state of the art high resolution imaging that identifies frank plaque haemorrhage, to a point whereby we are able to differentiate a much earlier stage of red blood cell infiltration that drives plaque inflammation.

Ferric Hb but not ferrous Hb products are responsible for generating an environment that promotes the oxidation of LDL. While the TBARS assay generated a destructive environment, with high temperatures and acidic reagents, this non specific test still showed that ferric Hb products produced more MDA than their ferrous counterparts. Additionally, the cisPnA assay that measures fluorescence quenching upon oxidation of lipid, showed a marked difference between intracellular and extracellular ferric Hb. Thus extracellular ferric Hb particularly generates an environment that is pro-oxidant. No significant changes in cumulative PnA fluorescence were seen with ferrous oHb or dHb products, indicating these products are not strongly associated with lipid oxidation. This reinforces the notion that ferric iron is able to commence a cascade of hydroxyl radical generation through the Fenton reaction.
The PnA data shows that extracellular mHb generates significantly more lipid oxidation than the intracellular products; however, mHb in both these environments has similar measures of r1. Therefore the T1 hyperintensity due to mHb is not solely restricted to an environment that causes lipid oxidation which may therefore diminish its ability to reflect at-risk plaque. However, it is known that in the absence of any chemical modifications, ferric haeme significantly degrades red blood cell membrane integrity, and the eventual fate of a static red blood cell is lysis\(^20\). Thus the intracellular mHb is destined to rapidly become extracellular, adding to the oxidative drive.

Some authors have proposed using T2* measures to evaluate the status of the RBC cellular membrane\(^26\). It has been suggested that there is a correlation between a low T2* value, indicative of intracellular blood products, and poor patient outcome. The evidence presented in this paper however would suggest the contrary, as intracellular products appear significantly less harmful than extracellular blood products owing to the barrier effect of the cell membrane against lipid oxidisation. The differentiation of intracellular and extracellular components using T2* likely reflects intact RBCs indicating fresh plaque haemorrhage, defining the start of a destabilisation event.

These results in combination suggest that ferric Hb is a species that needs tight biological regulation. The cellular membrane of the RBC, as well as the many endogenous scavenging molecules serve as primary and secondary defense mechanisms. However in the intraplaque environment, access to these molecules may be inhibited, or the concentration may simply not be sufficient to completely sequester the ferric iron molecules. This suggests that the detection and neutralisation of this pooled concentration of highly pro-oxidant ferric Hb would be of great clinical interest.

Ferric iron therefore appears to be a potential therapeutic target that is easily detectable using MRI. Chemical modification, either through chelation or reduction of the ferric mHb species, may be a method of inhibiting further mHb mediated lipid oxidation. However, therapeutic experiments with coincident histological data are presently difficult due to lack of good animal models that allow pre-clinical testing of novel therapeutic interventions.
The observations made are supportive of the iron hypothesis of atherosclerosis development. We have shown that the iron not only needs to be labile and accessible to the extracellular environment but the oxidised ferric form is required – a form that is readily detected using MRI. This raises the possibility of using similar imaging techniques in other diseases in which iron has been implicated such as: multiple sclerosis\textsuperscript{21}, Alzheimer’s disease\textsuperscript{22} as well as various cancers\textsuperscript{23-25}. The implication of localising these foci of active iron deposition with MRI may enable significantly earlier detection, and subsequent intervention.

One limitation of this study is that we have not considered the effects of T2* in the calculation of the relaxivity. We attempted to minimise T2* effects through using the shortest possible TE (effective TE at centre of k-space 5.0ms), however, at higher concentrations, the data could be biased by the loss of signal, possibly altering our measured relaxivity. Additionally, we have not explicitly demonstrated that the T\textsubscript{1} hyperintensity associated with MR detected IPH is ferric haemoglobin. However, previous studies\textsuperscript{17,18} have shown excellent correlation between the MR hyperintensity and histopathological presence of RBCs. The possible cause of the hyperintensity then is either mHb or a tenfold larger concentration of dHb or oHb. From our own observational data T\textsubscript{1} ranges from 300-400 milliseconds in signal hyperintensities at the carotid bifurcation at 3T. Using the calculated r1 value of approximately 0.3mM\textsuperscript{-1}s\textsuperscript{-1} and an unenhanced blood T\textsubscript{1} value of 1200ms, this range corresponds to a mHb concentration of approximately 9-15mM. The intraplaque concentrations required for the same measured T\textsubscript{1} with oHb or dHb would therefore be an order of magnitude higher which is likely a higher concentration than can be achieved \textit{in vivo}. Together, this evidence suggests that the predominant species present in these T\textsubscript{1} positive atherosclerotic plaques is ferric haeme – thus presenting a hostile, highly oxidative environment to incoming LDL molecules as we have shown.

In conclusion, we have demonstrated that ferric haeme is significantly more pro-oxidant than ferrous haeme. Additionally, we have shown that this ferric haeme has a much higher relaxivity than its ferrous counterparts. This evidence in tandem suggests that the
MR detected $T_1$ hyperintensity within the vessel wall is an endogenous biomarker of an intraplaque environment that is highly pro-oxidant and pro-atherogenic.
2.5 References


Chapter 3 Haptoglobin Phenotype Modulates MRI Detected Signal from Methaemoglobin

3.1 Introduction

In the previous chapter, it was shown that the signal hyperintensity associated with magnetic resonance detected IPH is related to an environment of increased oxidative stress. Furthermore, we know that IPH also contributes to the growth of the necrotic core, increases oxidative stress within the vessel wall and delivers iron to the plaque core in the form of haeme.

Owing to its toxicity, many endogenous protection mechanisms have evolved to modulate the effects of free iron. The first line of defence in vivo is the red blood cell membrane that sequesters Hb inside the RBC. This membrane allows essential exchange of oxygen and water, however, it inhibits large proteins from entering or escaping the cellular membrane. However, the eventual fate of a trapped RBC is lysis of the red cell membrane, spilling the iron rich haeme molecule into the surrounding environment.

The second line of defence behind the RBC membrane is the circulating blood proteins that bind particles that enter the blood stream. Many such proteins exist that non-specifically bind foreign bodies in circulation, however, the most specific for haemoglobin is Haptoglobin (Hp). Hp, originally described as anti-haemoglobin, appears to specifically bind extracellular Hb. This binding appears to modulate the reactivity of Fe$^{3+}$. The Hp gene is polymorphic in humans with two common alleles, Hp-1 and Hp-2. These two alleles appear to offer differing degrees of cardiovascular protection; those individuals with the Hp-2 allele appear to be at greater risk of cardiovascular events, especially when paired with diabetes, compared to individuals with only the Hp1 allele. Additionally, the Hp-2-2 phenotype is associated with an increased oxidative stress load within atherosclerotic plaque. Together, this data seems to indicate that patients possessing an Hp-2 allele are at increased risk for cardiovascular disease.
The Hp protein sequesters Fe\textsuperscript{3+} from the surrounding environments, modifying its ability to react with lipids. In the context of the Fe\textsuperscript{3+} associated MR signal hyperintensity, this sequestration may alter the ability of water to interact with the paramagnetic Hb-Fe\textsuperscript{3+} core, changing the MR properties. Therefore, we studied the MR signal behaviour of Fe\textsuperscript{3+} in the presence of the different Hp proteins. We hypothesise that the very large Hp proteins may alter the ability of surrounding water molecules to approach the paramagnetic core and therefore modulate the detected MR signal.

### 3.2 Methods

#### 3.2.1 Sample Preparation

Informed consent was obtained from N=6 volunteers to collect 30mL of fresh blood in this study approved by the research ethics board. Blood was collected and prepared as outlined in the previous chapter.

Hb-Fe\textsuperscript{3+} was prepared by incubating 50 mg nitric oxide donor diethylamine-NONOate (Sigma D5431) for every millilitre of Hb solution for at least 30 minutes at room temperature. Concentration of haemoglobin was calculated as detailed in Chapter 2. From this concentration, enough haemoglobin was added such that 1mM of ferric Hb was present after the dilution with the remaining test solutions. This blood product was added before the addition of any other test solutions.

Haptoglobin protein phenotypes Hp1-1 and Hp2-2 (Sigma Aldrich H0138 and H9762) were reconstituted in phosphate buffered saline immediately prior to each experiment and were used to create a 10.5μM stock solution. Hp was added to the sample tube prior to the addition of any blood products. To compare the protection efficiency of Hp, Desferrioxaine (DFO) (Novartis) a specific Fe\textsuperscript{3+} chelator was used as a control.

Samples were prepared in a 96 deep well plate with well volumes of 600μL. To determine the effect of blood proteins Hp-1-1, Hp-2-2, on lipid oxidation 0, 100, 200 and
300 μL of each of the haptoglobin stock solutions were added to the wells with a balance of phosphate buffered solution to make 600μL yielding concentrations of 1.7μM to 5.25μM Hp. As mentioned before, 1mM of Ferric Hb was already in the test well. 1mM hydrogen peroxide was then added to this solution.

3.2.2 Magnetic Relaxivity Measures

MR relaxometry parameters were measured in a 3T Philips Acheiva (Philips Medical Systems) using a 2D Look-Locker technique as described in the previous chapter\textsuperscript{13}. Percent change normalised to no Hp protein was calculated to ease comparisons between the two proteins.

3.2.3 Measuring Lipid Oxidation

To determine the lipid oxidation ability of Hb-Fe$^{3+}$ in the presence of Hp-1-1 and Hp-2-2 the TBARS assay was used as described in the previous chapter. The same volumes as described previously (100μL of sample, 100μL of sodium dodecyl sulfate and 50pg of purified LDL) were used in the assay.

3.2.4 Patient Haptoglobin Genotyping

To evaluate whether the effects seen in vitro also hold in vivo, N=46 patients consented to have their Hp phenotype correlated with their MR imaging in a study approved by the local research ethics board. Individuals were recruited consecutively from patients with prior diabetes volunteering for a neurovascular MRI with contrast between December 2008 and June of 2010. These patients had no previously known vascular disease and were otherwise asymptomatic for neurovascular symptoms. All study patients were imaged using a 3T Philips Acheiva scanner using an MRI pulse sequence previously validated to detect intraplaque haemorrhage\textsuperscript{14}. Pulse sequence parameters are displayed in Table 3.

MR images were read by radiologists blinded to the results of the Haptoglobin phenotype and MRIPH positivity or negativity was taken from the radiological report.
Blood samples were sent to a lab (D. Cole, University Health Network) specialising in DNA extraction for sequencing. The protocol used to determine the Hp phenotype is described below for completeness. DNA was extracted from blood as described previously\textsuperscript{15}. Part of the Hp gene was amplified in 10\textmu l polymerase chain reaction (PCR) solution containing 1X PCR buffer (Qiagen), 2X Q-solution (Qiagen), 0.3mM each of dNTPs, 25 ng genomic DNA, 0.05U HotStar HiFidelity DNA Polymerase (Qiagen), 2U of HotStarTaq\textsuperscript{®} Plus (Qiagen) and 0.4\mu M each of primers 5’-GGGGAGCTTGCCTTTCCATT-3’(forward) and 5’-GGCTGTCACTGCTGCGTAAA-3’ (reverse). The reaction was initiated by activation of the hot-start polymerases at 95\textdegree{}C for 2 min, followed by 35 cycles each of 10 sec at 94\textdegree{}C, 1 min at 62\textdegree{}C and 7min 30sec at 68\textdegree{}C. The PCR products were separated by 1\% agarose gel electrophoresis and visualized with UV illumination. The Hp-1-1 phenotype produced a 1920bp band and the Hp-2-2 phenotype a 3644bp band.

3.2.5 Statistical Analysis

Student’s t-test was used to determine difference between means. Pearson’s Correlation coefficient was calculated to determine the linearity of the relaxivity fit.
3.3 Results

3.3.1 Haptoglobin Proteins Show Different Ability to Prevent Lipid Oxidation

Figure 3.1 shows the iron specific chelator DFO significantly inhibits the formation of lipid oxidation products compared with unchelated controls (p<0.0005). The two phenotypes of Hp also demonstrate a measurable difference in TBARS attenuation compared with controls (both less than p<0.005). The Hp-2-2 protein allows more lipid oxidation than either Hp-1-1 or DFO (both less than p<0.005), whereas the Hp-1-1 protein is not significantly different from DFO (p>0.05).
The Hp-1-1 / Hb-Fe³⁺ complex has decreasing relaxivity with increasing concentrations of Hp-1-1. This decreased relaxivity would translate to a darker signal on

Figure 3.1: Normalised Thiobarbituric Acid Reactive Species (TBARS) Assay for Various Iron Binding Molecules

Upon addition of Desferrioxamine (DFO) a significant drop in TBARS is seen (p<0.0005) – this holds true for both Haptoglobin proteins (Hp1 and Hp2) (p<0.005). However, the decrease in TBARS production is not as dramatic for the Hp-2-2 protein as is for the Hp-1-1 protein, which is not statistically different from the addition of the DFO molecule.

3.3.2 MR Relaxivity Measures
Haptoglobin Phenotype Modulates MRI Detected Signal from Methaemoglobin

Conversely, the Hp-2-2 protein increases the relaxivity of the sample (See Figure 3.2). This increased relaxivity translates into a hyper intense signal if imaged using T₁ weighted imaging. The Pearson’s R² correlation coefficient for both of these fits is greater than 0.8, with actual correlation coefficients reported in the figures.
3.3.3 Patients with a Hp-2 Allele Have a Higher Rate of presentation of MRIPH Positive Disease Than Patients With Only Hp-1 allele

The small patient study generates a contingency table as shown in Table 2, with all four patients who were MRIPH positive having an Hp-2 allele. Furthermore, no patients with the Hp-1-1 phenotype presented with MRIPH. These results are not statistically significant with a Fisher's Exact test p value of 0.407. Taking the observed incidence rate from the Hp-2 group of 4 in 37 as the expected observation rate in the Hp-1, the
probability of observing a distribution this extreme of 0 cases of IPH in this cohort of 9 patients is only 0.357 = (33/37)^9.

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Table 2: Haptoglobin Phenotype Distribution with respect to MRIPH status

This 2x2 contingency table shows the Haptoglobin (Hp) status of patients and their MR status, either positive IPH signal detected (MRIPH+) or not (MRIPH-). Of the n=46 patients, only 4 of these were MRIPH positive, however all four had a Hp-2 allele (either Hp2-2, or Hp1-2). The remaining 33 patients presenting with a Hp-2 allele were negative for MRIPH, suggestive that Hp-2-2 status alone is not sufficient for plaque haemorrhage. However it appears to be one risk factor that determines which patients may go on to haemorrhage.

3.4 Discussion

We have shown that the two most dichotic phenotypes of Hp have different abilities to inhibit lipid oxidation with the Hp-1-1 phenotype appearing to be more effective than Hp-2-2. Additionally, these phenotypes differ in their appearance when observed using a clinical 3T MRI system with the Hp-1-1 phenotype inhibiting Fe^{3+} enhancement and Hp-2-2 phenotype increasing Fe^{3+} enhancement. This data supports
the hypothesis that patients with MRI detected IPH have a Hp phenotype that is less able to prevent iron mediated lipid oxidation thus increases plaque vulnerability.

Additionally, in a small group of patients, the appearance of hyper intense plaque haemorrhage was seen only in patients with a Hp-2 allele and not seen in patients presenting with the Hp1-1 phenotype. While this patient study is not statistically significant, it has a trend that would support the hypothesis that patients with Hp1-1 phenotype do not develop an MR detectable IPH.

*The interaction of Haemoglobin, Fe$^{3+}$ and the Hp Molecules*

Hp appears to change the magnetic relaxation properties of the Hb-Fe$^{3+}$ by interposing between surrounding water molecules and the core; separation between the two magnetic cores as small as three Angstroms can decrease the MR relaxation rate by nearly a factor of two$^{16}$. Our data for Hp-1-1 protein indicates that magnetic interactions are inhibited, suggesting the size of this Hp-1-1/Hb-Fe$^{3+}$ complex is of a scale that prevents water from tumbling close to this paramagnetic centre. Furthermore, the reduced TBARS verifies that not only are magnetic interactions inhibited, chemical interactions are also inhibited as the Fe$^{3+}$ core cannot interact with the LDL. Thus, in the presence of Hp-1-1, we may not observe MRI hyperintensity and LDL oxidation will be reduced. The Hp-2-2 data shows the opposite behaviour, with increasing concentrations of protein increasing T1 relaxivity. Further the TBARS data suggests that while there is a small reduction in lipid oxidation, this protein is inferior to Hp-1-1 at inhibiting Hb-Fe$^{3+}$ mediated iron oxidation.

These results suggest that the Hp-2-2 / Hb-Fe$^{3+}$ is of a form that allows external water continued access to the Hb-Fe$^{3+}$ core. Whereas Hp-1-1 proteins form stable monomers of approximately 400kDa, the Hp-2-2 protein forms cyclic polymers of varying sizes owing to the variability in the alpha subunit of the Hp protein$^{17}$. These polymers are as small as Hp-1-1 (400kDa), but can range in size up to 900kDa. The larger proteins appear to improperly bind the Hb-Fe$^{3+}$ complex and water molecules are still able to both physically as well as magnetically approach this iron core.
When the Hp2-2 complexes with Hb-Fe$^{3+}$, the resulting large molecule has a significantly increased correlation time compared to Hb-Fe$^{3+}$ alone. This large, slowly tumbling molecule appears to be a much more efficient relaxation agent than the smaller free tumbling Hb-Fe$^{3+}$ molecules. This increased relaxivity would lead to a more hyper intense signal on T$_1$ weighted MRI.

While the changes in relaxivity we show in this work are relatively modest (on the order of several %) the concentrations used in these experiments, in the micromolar regime, are several orders of magnitude less than circulating levels of Hp, which usually appear in the hundreds of micromolar to low millimolar regime. We would expect this larger concentration of protein to continue binding the free ferric haemoglobin, affecting the magnetic properties.

Also, the previous chapter used both TBARS and cis-PnA to determine the differences between the intra and extracellular blood products, here only TBARS is used. While TBARS is a non-specific for lipid oxidation, in the absence of confounding factors such as cellular membranes it provides an accurate approximation of the lipid oxidation potential of the chemicals assayed.

### 3.4.1 Haptoglobin’s Association With MRIPH

We have also shown that in this small group of patients, MRIPH positivity was associated with the Hp-2 allele. None of the patients with Hp-1-1 had MR detected IPH, suggestive that this may be a phenotype that is associated with stable plaque morphology.

The results of this small study show that Hp phenotype mediates Hb-Fe$^{3+}$ signal intensity; this effect may be owing to the MR properties we have shown in this paper or may be due to the previously shown inability of Hp-2-2 to access the plaque environment. Owing to the large size of the Hp-2-2 protein, ingress of this protein into the plaque core may be limited$^8$. Regardless of the cause, either MR detected IPH, or
Hp-2 allele appear to predispose patients to future ischemic events. Absence of these markers may be a sign of more benign plaque morphology.

We have shown in vitro that various iron binding agents, both endogenous and exogenous, can modify the aggressive lipid oxidation environment found in bleeds such as intraplaque haemorrhage. Not only do these molecules have a differential effect in terms of ability to inhibit this proxidant state, we see a marked difference in how they behave in an MRI environment.

3.4.2 Implications for Patient Management

The Hp phenotype of a patient may be an indicator of patient risk arising from the oxidative effects caused by intraplaque haemorrhage. From the data presented here, there is a suggestion that those with an Hp-1-1 phenotype may not develop a hyper intense MRIPH plaque. From previous longitudinal studies, absence of this imaging biomarker suggests that the patient has a stable plaque phenotype.

On the other hand, our in vitro data suggests that binding of Hb-Fe$^{3+}$ with Hp-2-2 would increase the conspicuity of this IPH on our MR images. This in vitro data suggests that MR detected IPH may be more than an indicator of future stroke potential. This MRI imaging biomarker may possibly be an indicator of a patient lacking endogenous Hp protection and at high risk of systemic cardiovascular disease.

Conversely, knowing the patient’s Hp phenotype, we may be able to subselect a group of patients at risk of developing MR positive IPH. Again, based on the small numbers in this study, it appears to be this subset of patients who go on to develop future clinical symptoms, and the Hp phenotype may identify patients that require more thorough work up for cardiovascular risk.

3.4.3 Future Direction

As suggested previously$^{18}$, inhibition of the Fe$^{3+}$ may be a possible therapeutic for patients with atherosclerotic disease. To date, no chelator has been shown to reduce
plaque size or volume in a patient cohort with atherosclerotic disease. This lack of data may be due to the non-selective group of patients with generalised carotid stenosis. Many patients will have carotid stenosis without concomitant IPH. Subselecting a group of patients within this cohort, ones who have an active Fe$^{3+}$ plaque core as defined by MRIPH, may more accurately target those patients for whom chelation therapy would be successful.

3.5 Conclusions

We have shown that modulation of the iron environment in vitro via the use of chelating agents can significantly alter the ability of iron to oxidise LDL. Furthermore, we have shown that this modulation is accompanied by a detectable change on a clinical MR scanner, with only the Hp-2-2 / Hb-Fe$^{3+}$ complex having a high relaxivity and the Hp1-1/Hb-Fe$^{3+}$ complex having a much lower MR relaxivity.
3.6 References


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Table 3 Pulse Sequence Parameters
Chapter 4
Detecting Methaemoglobin Outside Clinical Magnetic Resonance Environment

4.1 Introduction

As has been discussed in earlier chapters of this thesis, iron deposition secondary to intraplaque haemorrhage appears to identify patients at risk. From a clinical point of view, patients presenting with MR detected plaque haemorrhage appear to be at greater risk of future cerebrovascular clinical symptoms. The in vitro work presented in this thesis has shown that ferric iron generates an oxidative environment, causing an increased rate of LDL oxidation. These two facts taken in tandem are highly suggestive then that the presence of plaque haemorrhage is not simply a secondary manifestation of plaque complication. Rather, this oxidative environment generated by the deposition of the plaque is a driving force behind increased lipid oxidation which in turn leads to greater amount of inflammation. This is hypothesised to increase neo-vascular growth, increasing the amount of blood to the area thus leading to greater blood deposition, which in turn increases the amount of plaque haemorrhage\(^1\). Small amounts of uncontrolled Fe\(^{3+}\) may be the required stimulus for a stable plaque to enter a phase of rapid growth.

To further supplement this argument, recent evidence has shown that IPH is a risk factor predisposing patients to global vascular disease\(^2\). This study followed patients after endarterectomy and showed that neovessels and intraplaque haemorrhage were the only histological correlates of future global events in these patients. Patients who had IPH or plaque neovessel in the carotid endarterectomy specimens had a hazard ratio for recurring stroke 1.7 times those presenting without plaque haemorrhage. This suggests that detecting IPH in a patient anywhere in their vascular tree marks not only a single vulnerable plaque, but identifies a vulnerable patient.
It would seem beneficial then to be able to screen patient populations for this marker of globalised vascular disease. This however, is unlikely to happen in the MR environment as access issues associated with cost and timing are prohibitive to make this technique widespread. For this paradigm to exist, a high sensitivity test needs to be developed that could pre-select patients at risk for a follow up MR exam. Unfortunately, the image hyperintensity generated by IPH is not a unique signal; the proton enhancement it causes is not specific for IPH and signal hyperintensity is merely a surrogate indicator of the presence of some relaxation agent. Improvements in the sensitivity may lie in looking beyond the secondary effect of proton relaxation enhancement generated by Fe$^{3+}$ and looking directly at the paramagnetic electron signal.

Similar to the proton resonance, electron paramagnetic resonance relies on the absorption and emission of a specific frequency of energy. However, the key difference is that very few molecules in the human body have a stable unpaired electron required to generate an EPR signal. Often molecules with unpaired electrons are associated with free radicals, which quickly react with other compounds nearby to enter a lower energy state where their orbital electrons are paired. Biological molecules of interest with this type of behaviour include the vasodilating molecule nitric oxide. This reaction is difficult to capture as NO lifetime is exceedingly short. Conversely, Fe$^{3+}$ is unique in this regard; in observational studies done in our lab, the signal hyperintensity associated with deep venous thrombosis lasts for days whereas the hyperintensity associated with carotid IPH has persisted for months$^3$.

The EPR resonance is governed by the equation

$$\gamma_e = g_e \mu_o B_o$$

where $\gamma_e$ is the electron gyromagnetic ratio, $\mu_o$ the Bohr Magneton and $B_o$ the main magnetic field with $g_e$ the unitless correction factor for the electron. For a free electron $g_e$ is approximately equal to 2 (2.0023193 from the National Institute of Standards and Technology) yielding a gyromagnetic ratio of approximately 28.024GHz/T – approximately 659 times greater than the proton gyromagnetic ratio. Thus at current day
clinical magnetic field strengths of 1.5T and 3T, the resonance frequencies for an EPR signal would be 42 and 84GHz respectively. While generating these frequencies is not beyond the realm of possibility, the penetration depth of this wavelength of RF is on the order of a few millimetres. This makes exciting the tissue at any depth greater than this nearly impossible without superficial heating. However, if on the other hand, we look at generating RF within the range of clinical frequencies we are using today (64MHz and 128MHz) the penetration depth would be significantly less of an issue but the magnetic field strength would have to decrease by a factor of 659 (to approximately 2-5 mT).

With this decrease in field strength is a concomitant decrease in the amount of signal. However, if the modality is shifted away from the imaging of EPR to purely a detection of EPR, major gains can be made in relation to signal bandwidth. The intrinsic noise in MR is given by

\[ N = \sqrt{4kTR\Delta f} \]  

(13)

Where \( k \) is the Boltzman Constant, \( T \) the temperature, \( R \) the resistance of the receiver coil and \( \Delta f \) is the receiver bandwidth. Typical clinical MR imaging hardware has a receive bandwidth of tens of kilohertz to hundreds of kilohertz. This large bandwidth is due in part to the imaging gradients by which MR spatially encodes information. However, if we sacrifice our ability to spatially localise the signal and focus purely on the detection of this endogenous EPR signal, we can limit the receive bandwidth to several Hertz. Signal gains on the same order of magnitude lost to the decreased magnetic field can be achieved.

We hypothesise that low field EPR can be used to detect ferric haemoglobin products in vitro. We aim to produce a bench top system that can detect this signal and characterise its properties to better guide a future design of a low field portable system.
4.2  Methods

4.2.1  Generation of a Swept Magnetic Field
Figure 4.1 Photograph of Benchtop System

An overview photograph of the benchtop system described in this chapter. The primary components in the photograph are labeled with letters as follows:

A) A pair of High Current DC power supplies
B) Two 30cm radius coils wired in a Helmholz configuration
C) Function Generator stack
D) Transistor Amplifier with heavy heat sink containing glycol bath
E) Sample Shield (removed from magnet centre)
F) Cascaded preamplifier with shielding removed
G) Readout oscilloscope
Two 30 cm coils with a total inductance of approximately 40 mH were placed in series in a Helmholtz configuration. To optimise the homogeneity of the magnetic field owing to the positioning of the coils, the following procedure was performed. The coils were attached to a DC power supply and current was limited to 4 Amperes. All magnetic field measurements were made with a hand held Gauss meter (F.W. Bell 048) placed inside the central volume and swept by hand through the iso-centre of the magnet. The spacing and alignment of the two magnetic field coils were adjusted iteratively until a region of +/- 0.5 Gauss was obtained in a central 2 cm section of the coil.

Generating a stable magnetic field within tenths of a Gauss is problematic as current regulation alone is insufficient to guarantee a temporally and spatially stationary magnetic field. In our system, changes in the driving current of one milliampere leads to changes of approximately ten micro Tesla; this change represents a shift in the resonant frequency of several hundred kilohertz. Additionally, resistive losses in the coil cause temperature changes in the coil. This also alters the reactance of the coil further adding to problems in field regulation. Thus, instead of a stable stationary field, the designed field was swept over a range of interest. A power Darlington pair transistor (MJ11014) was used in class A mode to drive the magnetic field in a sinusoidal pattern (see Figure 4.3 left). Although electrically inefficient, class A operation was chosen to remove any cross over distortion obtained from a driver operated in a push-pull topology.

An arbitrary wave generator (Tektronic AWG420) was used to generate a waveform with a 200mV peak to peak 60Hz sine wave on a 0.1 Hz sine carrier ranging from 3 to 6 volts. When connected to the Helmholtz coil, a swept magnetic field of approximately 0.1 milliTesla and an offset ranging from 1 to 4 milliTesla was created (see Figure 4.2)
4.2.2 Absorption Apparatus

To detect the presence electron paramagnetic resonance, an absorption apparatus similar to Bloch\(^5\) was used.

Figure 4.2: Magnetic Field over Time

The magnetic field generated by the controller unit as a function of time. The amplitude and the frequency of the superimposed sine wave are merely illustrative and not to scale of the experiment described. The thick blue line indicates the driven field, whereas the horizontal blue line indicates the magnetic field where resonance is expected. The two boxes indicate two different areas where resonance absorption would take effect.
The schematic diagram for this circuit can be found in Figure 4.3 right.

Figure 4.3: Schematic of Magnet Driver and Receiver Chain

On the left is depicted a schematic of the magnetic field driving circuit and on the right, the receive chain. Transistor is a Darlington Par MJ11014 transistor, a flyback diode is installed in parallel with the Helmholtz coil, depicted as an inductor in the schematic. The voltage supply is a DC source capable of supplying 15 amps of current.

The right section shows the receive chain with the detection coil (inductor left) tapped after the first turn and a signal is injected at this point from the signal generator. The tunable capacitor in parallel allows tuning (but no matching) of the coil. This is followed by a peak / trough detector circuit and then four stages of amplification provided by the LMH6624 chip configured in low pass mode with each stage giving a gain of 10 and a cut off of approximately 1 kHz. The section denoted by parenthesis on the right side of the schematic is cascaded four times.

#18 magnet wire was wound into a 6 turn solenoid around a BD Vacutainer tube, 1.25cm in diameter over approximately 2 cm. A 25-75pF variable capacitor was placed in parallel to adjust the resonance of the circuit, which was tuneable from approximately
50MHz to 100MHz as verified on a network analyzer. The coil was left tuned to 85MHz, resulting in an expected absorption field of approximately 2.6mT. The coil was tapped one turn from the grounded end and a low power sinusoidal signal was injected at this point.

The RF signal was produced from a HP 8656B signal generator at 1V amplitude. Following the coil, a low forward voltage drop Schottky diode detector was used to detect the envelope of the absorption signal. All of the above were housed in a grounded metal case of approximately 5cm x 10cm x 3cm in dimension (E). The signal from the diode detector was then fed into an ultra low noise (0.98nV / Hz) LMH6624 operational amplifier with four identical stages of approximately 10 times gain for a total of 80dB of gain, housed inside an aluminum, grounded case (Figure 4.1F).

The signal driving the magnetic field was fed into an oscilloscope (Figure 4.1G) in XY mode to control the horizontal axis and the output of the amplifier was used to drive the vertical deflection.

4.2.3 Sample Preparation

Samples were prepared as previously described in Chapter 2. Briefly, fresh blood was drawn into green topped BD Vacutainers, red cells were separated by centrifugation, and lysed using 5 volumes of distilled water. The concentration of RBCs was determined using Drabkin's reagent. Ferric haemoglobin was created by incubation with NO donating agent DEA-NONOate.

A stock solution of 10mM Fe$$^{3+}$$ Hb was made and diluted to 1mM and 100uM to determine the sensitivity of our system to the EPR signal of Fe$$^{3+}$$ haemoglobin. 3mL of each of the solutions was placed into a sample container and the amplitude of the signal deflection was recorded. A linear fit was used to determine the amount of signal in mV per concentration. Pearson’s Correlation coefficient was calculated to determine the linearity of the data.
4.3 Results

Figure 4.4 shows a photograph of the oscilloscope display when a sample of ferrous oxyhaemoglobin is placed in the magnetic field, whereas Figure 4.5 shows a photograph of the display when ferric haemoglobin is placed in the field. The absorption peak appears as the field sweeps into resonance and moves from low field (left) to high field (right).

![Figure 4.4 No Absorption from Ferrous Haemoglobin](image)

Photograph of the Oscilloscope display when a sample of ferrous hemoglobin is placed in the magnetic field. Oscilloscope scale is 50mV per division horizontally, 10mV per division vertically. The horizontal display translates to approximately 2.8 MHz per division.
Figure 4.6 shows a plot of the signal intensity as a function of the concentration of Ferric Haemoglobin. The intercept of the linear fit is approximately 2.7 mV, which is in the noise floor of our system.

Figure 4.5 Resonance absorption with Ferric Haemoglobin

Two photographs taken several seconds apart, showing the oscilloscope output after 10mM ferric haemoglobin sample is placed in the magnetic field. The clearly visible resonance absorption sweeps from the right side of the screen to the left and back as the field moves in and out of resonance. Oscilloscope scale is 50mV per division horizontally and 10mV per division vertically. Again, the horizontal scale translates to approximately 2.8MHz per division. As the detector is displaying the voltage signal driving the magnetic field and not the current in the magnetic field, the display lags the actual B field by 90 degrees and the expected double resonance peak is not visualized.
4.4 Discussion

We have shown, that in a bench top system, the EPR absorption signal is detectable to small concentrations of approximately 0.1 mM.

To determine the relative sensitivity of this system to the current implementation of IPH detection using proton MR, it is useful to compare the sensitivity of the two techniques. Since there is a strong background proton signal from the surrounding tissues, a focal spot of signal hyperintensity of 1.5 to 2 times is usually used as a cut off for defining the presence of plaque haemorrhage. Using the values of relaxivity calculated in earlier work (Ch 2), the proton relaxivity of ferric haemoglobin is approximately 0.32/mM/s. Our initial work into the measurement of vessel wall $T_1$ shows us that healthy vessel wall has a value of approximately 900-1000ms at 3 Tesla. Ignoring transverse relaxation, the signal equation from a spoiled fast gradient echo sequence is proportional to

![Signal Amplitude vs. Concentration](image)

Figure 4.6 Plot of Signal Sensitivity vs. Haemoglobin Concentration

The signal amplitude of the deflection curve is plotted as a function of haemoglobin concentration for three concentrations. The fit has a slope of 0.75mV per mM and a zero intercept of approximately 2.7mV.
\[
S \approx \frac{\sin \theta (1 - e^{-\frac{TR}{T_1}})}{1 - \cos \theta e^{-\frac{TR}{T_1}}}
\]

Where $TR$ is the repetition time and $\theta$ the flip angle. Using typical values for the acquisition sequence of $TR=10\text{ms}$, and $\theta = 15^\circ$ degrees we calculate that for a 1.5 times signal difference, the effective $T_1$ would have to be near 500ms to 570ms equating to concentrations of 2.7 to 2.3 mM respectively. For a doubling of signal, $T_1$'s of 300-350 or concentrations of 5.6 to 6.7mM would be required.

While on the outset it seems that the detection system proposed has sensitivity 10 fold greater than that shown in proton MR, the figures need to be compared in absolute terms. One great advantage of proton MR in the detection of paramagnetic contrast agents is that one agent has the capability of relaxing many surrounding water molecules, thus greatly enhancing the sensitivity of proton MR. The calculation done for the proton MR above is on a per voxel basis – current acquisition voxel sizes are approximately 0.5 x 0.5 x 1.0 mm yielding a voxel volume of 0.25mm$^3$. While it is questionable to assume that the identification of positive IPH would be made on the detection of one single positive voxel, we will use that number in our discussion for now. Even at the largest concentrations discussed above, proton MR is sensitive to approximately 500 micromoles of ferric haemoglobin in this 0.25mm$^3$ voxel.

Conversely, the measurement volume over which the EPR system was sensitive was over three orders of magnitude higher (2cm vertical and a 1.25cm diameter) yielding a voxel volume of approximately 780mm$^3$. At its most sensitive, our bench top system is detecting 80 millimoles, or approximately 150 times less sensitive than the proton MR system.
4.4.1 Improvements in System Sensitivity

To make a feasible system, the sensitivity of the receiver needs to be increased several fold – below are proposed several methods to achieve this improvement. We will first look at replacing hardware within the receive chain, including modification of some of the receive chain to improve narrow band performance. Also, issues of field homogeneity will be addressed.

4.4.1.1 Hardware Receive Chain

Replacing the intrinsically noisy operational amplifiers with specially designed low noise amplifiers may yield a significant improvement in receiver performance. Noise figure, the ratio of the SNR out to the SNR in to the amplifier, is a figure of merit that is often quoted for amplification systems. For the operational amplifiers used in this implementation, the noise figure is usually quoted around 10dB. While considered “low noise” for an operational amplifier, these are still exceedingly inadequate amplifiers for very low signal detection such as described herein. Specially designed preamplifiers such as the Signal Recovery 5184 have quoted noise figures of approximately 0.1dB for an impedance matched coil in our frequency range of interest. This alone has the possibility to greatly decrease the noise floor in the detection system by nearly 10dB, easily lowering the lowest detectable limit to MRI detectable levels.

Another technique for signal improvement involves averaging the incoming data to reduce the noise floor of the system. The random noise contributions will, over a large number of samples, cancel out, whereas the data will average to form a coherent signal. However, this may rely on extremely well regulated magnetic field generation for the signal to coherently average. This in itself may pose a greater difficulty than improving the SNR of the signal. The signal shown above is approximately 1/8 of the sweep width, which was measured to be 0.1mT – thus shifts as small as 10µT are sufficient to move the absorption peak to completely obscure it in the noise.

Alternatively, as alluded to earlier, the bandwidth of the readout system has a huge influence on the signal to noise ratio of the detected data and further reducing the
bandwidth may further reduce the noise floor. In our case, we have used a simple 1st order low pass filter with an elbow frequency of approximately 1 kHz. This non-ideal filter has a roll off of only approximately 3dB per octave and thus the equivalent noise bandwidth is significantly larger than this 1kHz specified. One method of obtaining a very narrow bandwidth is to demodulate the signal and then steeply low pass filter this signal near its DC value. This lock-in type amplification will produce a time varying DC signal and time constants of 8-10 seconds are readily achieved with simple circuit components. Reducing the noise bandwidth from approximately 2kHz to 0.1 Hz gives a significant (on the order of 100 times) signal improvement. This concept is very similar to the averaging as described above, however the waveform shape and absorption peak are discarded to obtain only the DC amplitude proportional to the amount of deflection seen of the EPR signal over the acquisition bandwidth. Also, regardless of field drift, the absorption peak will still be locked into the sweeping of the magnetic field.

4.4.1.2 Magnetic Field Homogeneity

In the system described herein, the magnetic field homogeneity was measured to be approximately +/- 0.02 mT, or expressed as a ratio of the maximum magnetic field, approximately 2 parts per thousand. Compared to commercially available clinical strength systems that have magnetic field homogeneities quoted in the parts per billion, significant increases in peak signal strength can be had if even marginal increases in magnetic field strength are achieved. Using careful arrangement of permanent magnet systems, unilateral MRI researchers have been able to obtain homogeneity in these external fields of several ppm\(^{5-8}\)

4.5 Conclusions

We have introduced the idea of seeking the electron paramagnetic resonance signal from ferric haemoglobin as a unique identifier. While the design as implemented is likely insufficient to detect in vitro concentrations of ferric haemoglobin it guides the design of future implementations by giving relative sensitivity as well as areas for
improvement. Calculations seem to indicate that the signal originating from purely the Ferric haemoglobin may be sufficient to be detectable outside a high field MR system.
4.6 References


Chapter 5 Conclusions and Future Work

5.1 Summary

The data presented in this thesis support the hypothesis that the source of signal hyperintensity visualised in MRIPH may not be merely a marker of advanced atherosclerotic disease, but possibly a driver of the atherosclerotic process through increasing lipid oxidation.

In chapter 2, ferric haemoglobin was shown to be a potent transformer of LDL into its atherogenic oxidized form. This ferric haemoglobin component is the only form that could attain reasonable concentrations required to generate signal hyperintensities seen in clinical MRI. These data suggest that the MR detected hyperintensity associated with IPH is more than simply a marker of disease; this biomarker may identify a pro-inflammatory, pro-atherogenic source. This pro-atherogenic source may lead to the increased morbidity seen in previous natural history trials of MR detected IPH. Furthermore, this work identifies ferric haemoglobin as a potential target at which therapeutics could be directed.

In Chapter 3, the first steps towards directing therapeutics at iron were explored. Endogenous and exogenous inhibitors of ferric iron mediated lipid oxidation were studied in vitro. We demonstrated that all three chelators tested -- desferrioxamine, haptoglobin 1-1 and 2-2 -- offered differing degrees of protection against iron mediated lipid oxidation. Furthermore, the two haptoglobin proteins show differential effects on the MRI signal with one (Hp1-1) quenching the MR signal enhancement by Fe$^{3+}$, and the other (Hp2-2) enhancing this same signal. These data suggest that those presenting with MRIPH may be of a Hp-2-2 phenotype, which in turn suggests there is a subset population that may be at greater risk to the oxidative effects posed by free Fe$^{3+}$. Furthermore, it was shown in vitro that chelation of this ferric iron using DFO can inhibit the deleterious effects of free ferric iron, opening an avenue of exploration into reversing
the local pockets of reactive oxygen species (ROS), and possibly slowing the progression of atherosclerosis.

In Chapter 4, a detection technique using electron paramagnetic resonance was proposed. Again, using in vitro studies, it was shown that a distinctive signal could be detected from the ferric blood whereas no distinguishable signal could be detected from its ferrous counterparts. Unlike detecting the enhancement of the proton signal where large levels of contaminating “noise” from other biological tissues mask the enhancement caused by the presence of ferric iron, measuring the paramagnetic resonance directly examines a unique biological entity with very little background signal.

We have shown the cause of MR hyperintensity associated with IPH is not merely secondary to plaque progression, but possibly a potent, pro-atherogenic source. Furthermore we have also demonstrated chelation of these products inhibits oxidation, and may mitigate these pro-atherogenic effects. We have shown an imaging phenotype with data suggestive that it may be sensitive to a genotype. Finally we have demonstrated a bench top system that is capable of detecting the ferric iron paramagnetic resonance associated with plaque haemorrhage.

5.2 Future Work

The data shown in this thesis raise several questions and avenues of continued research. First, we have shown that the MR signal hyperintensity on T₁ weighted fat and flow suppressed imaging is likely indicative of a ferric iron component generating a lipid oxidising environment. As described earlier, this environment is not exclusive to the field of atherosclerosis; iron mediated hydroxyl generation has been implicated in a host of human diseases. Investigation into different diseases in which this signal hyperintensity may be a precursor of later disease will be discussed. Second, we have also shown that the Hp protein appears to alter the MR signal. The Hp-2-2 protein was shown to be less capable of attenuating ferric iron mediated lipid oxidation, as well, it increases the relaxivity of Hb-Fe³. This may indicate that only in a subset of patients do we see this signal hyperintensity that is correlated with negative future clinical outcomes. Third, we
have shown detection mechanisms looking specifically at the EPR signal from Hb- Fe\(^{3+}\). These studies need to be extended to more realistic situations of disease and the data from these studies need to be extended into devices that are more clinically tolerable. Lastly, the idea that this Fe\(^{3+}\) induced inflammation can be diminished by applying pharmacological agents that are capable of chelating or removing the Fe\(^{3+}\) core will be discussed.

5.2.1 Iron Mediated Damage in Other Diseases

As previously discussed, iron has been implicated in many forms of disease ranging from the subject germane to this thesis, atherosclerosis, to diseases as varied as Alzheimer’s, cancer, and – the subject of much recent attention-- multiple sclerosis. While this technique has been shown to detect complicated plaque in the form of IPH in many arterial sites\(^1\), this technique has also been used to detect deep venous thrombi\(^2\) as well as their emboli\(^3\) and endometrioma\(^4\).

5.3 Early Detection and Screening for Vulnerable Plaque

One goal of this work is to find a patient base that is vulnerable to this early indicator of future disease, with the intent of administering an intervention (either pharmacological or surgical) that would ward off clinical events. The section below discusses methods of extending some of the findings in this thesis to wider based preventative screening.

5.3.1 Patient Selection Based on Haptoglobin Phenotype

We have suggested that the Haptoglobin phenotype modulates MR signal intensity. This has been demonstrated in vitro, in well controlled circumstances. These data suggest that patients who have the Hp-1-1 phenotype are not only protected from the effects of iron enhanced lipid oxidation, but that the signal hyperintensity associated with intraplaque haemorrhage signal may be muted, or not detectable compared to those of Hp-2-2 phenotype. The small patient study done in this thesis also shows some
promising trends towards the hypothesis that MRIPH presents more often in Hp-2-2 patients than Hp-1-2 and Hp-1-1 patients. However, this study needs to be repeated with larger sample sizes. While our results are not statistically significant at the $p=0.3$ level, there are very few patients presenting in the MRIPH positive column ($n=4$). Even a single patient presenting with MRIPH with Hp-1-2 or Hp-1-1 would dramatically alter our results. Furthermore, the representation of MRIPH is low in this subset of patients; anecdotally, we see MRIPH in approximately 30% of the patient population being followed for neuroischaemic symptoms. There are patients in our study subgroup that were referred for non-specific neuroischaemic symptoms (i.e. headache, dizziness) and have non-significant carotid disease either as measured by MR angiogram, or by MRIPH. Thus patients previously known to have MRIPH would be an ideal group to recall for blood testing and follow up MRI. These could be compared to a stenosis and age matched group previously imaged with MRI who are known to be MRIPH negative.

As detailed already in Chapter 3, we have collaborated with a group to determine the Haptoglobin phenotype of a patient. This is done through extraction and sequencing a portion of the patient’s DNA. However, patient populations have become increasingly uneasy regarding genetic sequencing and testing. We have also done some preliminary work to determine patient haptoglobin type based on the protein itself in circulation.

Owing to their abundance in circulation, the Hp proteins present in sufficient concentrations to be visualised directly. Since they have significantly different sizes and conformations (400kDa Hp-1-1 to nearly 900kDa for Hp-2-2), the two alleles of Hp are easily separable using a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) assay. The technique previously reported was optimised for our system and equipment and is reported below for completeness. 6 mL of blood was withdrawn from the patient using a lithium heparin vacutainer through a large gauge catheter (up to 18 gauge, but as small as 20 gauge) inserted for MRI contrast administration. The patient’s red blood cells were first separated and packed through low speed (1500g) centrifugation. The supernatant plasma (approximately 3mL) was set aside and the remaining red cells were washed twice in three volumes of phosphate buffered saline. Nine volumes of double distilled water were then added to the red cell pack to lyse the
red cells and the solution centrifuged at 10,000g for 10 minutes. 500μL of the resulting supernatant was selected and added to the 3mL of plasma. This solution was added to an equal volume of sample buffer containing 125 mM TrisBase pH 6.8, 600μL glycerol and 3μL bromophenol blue.

The PAGE gels were mixed to create two different concentrations, a 4% stacking gel (29:1 acrylamide / bis-acrylamide) pH 6.8 and a 6% separating gel with the same ratio of acrylamide / bis-acrylamide pH 8.8. Electrophoresis was performed in a (MiniPROTEAN, Bio Rad) electrophoresis box at a constant voltage of 250 V for two hours. The gels were then transferred to a glass plate containing a buffer composed of 0.2 g of dimethoxybenzidine and 100 mL of 0.9 mol/L acetic acid. After two additional hours of incubation in the dark, 5mL of freshly prepared 3% hydrogen peroxide was mixed with the solution covering the gel. Bands became visible within approximately fifteen minutes after the addition of hydrogen peroxide and gels were documented with a digital photograph. Figure 5.1 shows an example of a patient with Hp-2-2 phenotype. A banding pattern is evident in the leftmost column where Hp has been incubated with the Hb solution.
This finding might be added to a battery of tests to obtain a cardiovascular risk profile tailored for each patient. This general blood testing prior to administering expensive imaging tests, of which MR is one, may direct our limited resources to patients who may most benefit from them. If these findings were to hold true in large scale studies, simple blood screening may be able to identify those patients who would
benefit from having intensive follow up using imaging, and would allow the health care provider to better channel limited resources. Additionally, the finding of an absence of IPH, indicative of stable plaques could possibly be reduced by blood testing of haptoglobin phenotypes 1-1 and 1-2.

5.4 Improvement of Detection Systems

In Chapter 4 a bench top system was used to demonstrate the feasibility of detecting Hb-Fe$^{3+}$ outside of an MRI environment. Before experiments can be attempted to correlate the findings between the proposed detection system and findings with MRI, prototypes more amenable to clinical implementation will need to be developed. Below are two possible embodiments of this idea in clinically useful devices.

5.4.1 Catheter Lab Implementation

As the system stands currently, very few modifications would have to be done for the quick translation of the bench top system into a device that would be useable in a catheterisation procedure. Currently, X-ray fluoroscopy is limited to lumenography. It is however, the gold standard by which image guided minimally invasive interventions are currently done. The high resolution of this technique allows the clinician to detect small amounts of abnormal narrowing, and the real time feedback allows the detection of abnormal vessel elasticity, possibly indicating an early stage of disease. However, this technique is unable to characterise the atherosclerotic plaques that it can so easily detect, and in the scenario of an artery with many lesions, an interventionalist may be forced to either treat via stenting many non-problematic lesions to stent correctly the high risk lesion. This increased stenting has its negative effects as it damages endothelium and could promote in-stent restenosis. Ideally treating only the problematic lesion would be desirable.

By giving some information whether or not a suspect plaque has sufficient Hb-Fe$^{3+}$ to give rise to a signal may be useful in determining the problematic lesions after
they are detected by X-ray. The main magnetic field coils of the system as it is currently implemented could be positioned around the head of a C-arm fluoroscopy unit thereby enabling standard X-ray imaging during the interventional procedure. Positioned correctly, a region of sufficiently uniform magnetic field could be generated – and the co-ordinates of this region with respect to the X-ray fluoroscopy image would remain fixed. A detector coil, such as those designed by interventional MRI researchers\textsuperscript{7} could be placed at the end of the catheter and the readout electronics fed back outside the patient. The interventionalist then only needs to manoeuvre the catheter into the anatomy of interest and then reposition the head of the fluoroscopy unit such that the catheter tip is encompassed in the region of homogenous magnetic field. The sweeping magnetic field could then be engaged, and a monitor would display the absorption peak. Alternately, the size of the absorption peak which appears to be nearly linear with the concentration of Hb-Fe\textsuperscript{3+} could be read out and gray scale intensity could be superimposed on the X-ray image depicting the amount of detected Hb-Fe\textsuperscript{3+} signal.

Advantages of this system include a very small, receiver coil located very near the anatomy of interest. The size of the coil minimises the volume over which noise will be picked up.

5.4.2 Handheld Detectors

While the catheterisation lab is an attractive arena for the implementation of such a device owing to the potential impact on patient management, the thrust of this work was to develop methods of detecting plaque haemorrhage in a population screening setting. This necessitates a non-invasive procedure. The primary tool of vascular disease staging continues to be Doppler ultrasound. Given the relatively low cost, real time imaging and the clinically validated metrics (e.g. intimal medial thickness) of this modality, it will continue to dominate vascular work up. However, as alluded to previously, this imaging modality does not have the ability to detect intraplaque haemorrhage sensitively.
We hypothesise that adding a Fe$^{3+}$ detection system to the head of a standard ultrasound probe will allow for more accurate characterisation of the plaques detected real time by Doppler. By placing three permanent, rare earth magnets parallel to each other but perpendicular to the face of the ultrasound probe, a small, homogenous region of magnetic field can be produced as detailed in Figure 5.2 [Marble].

![Magnetic Field Generated by 3 Permanent Magnets](image)

**Figure 5.2:** Magnetic Field Generated by 3 Permanent Magnets

Left, is a top view of a set of three magnets (denoted by arrows) and acrylic spacers. By varying the position of the central magnet, the region of homogeneity (denoted in green) can be repositioned. A region of interest, denoted by the dashed box, is enlarged in the right of the figure. Here we see the magnetic field vectors plot on a color map of the intensity of the magnetic field. In this design, a target of 2.5mT was used, and a region of homogeneity of approximately 1 part per thousand over approximately 1cm in diameter can be achieved.

Using the arrangement detailed in the diagram, a small loop coil could be placed parallel to the front face of the Ultrasound transducer. The magnetic field is also parallel...
to the front face of the transducer thus any excited magnetization could be detected. Again, a small area is localised owing to the limited area of uniformity of the surrounding regions. This area could be highlighted on the Doppler display and a color map intensity of the detected Fe$^{3+}$ signal could be overlaid. The difference in this system compared to the system described in the catheter lab is that the region of homogeneity has some small freedom of range; by moving the middle magnet forwards or backwards, the region of homogeneity shifts. Mapping this region of homogeneity, one would be able to generate several ROIs in which Fe$^{3+}$ content could be detected by raster scanning these ROIs individually.

Furthermore, wrapping these permanent magnet arrays in loops of wire, a fluctuating magnetic field could be generated, again controlled by an external current source. The current required to drive this field would be substantially less than those detailed in the previous chapter as the main magnetic field is provided by the rare earth magnets. The fluctuating field of several hundred micro Tesla would be easily supplied by several amperes through a coil of multiple windings surrounding these permanent magnets.

We hypothesise that the added information given by an Hb-Fe$^{3+}$ detection mechanism at the tip of the ultrasound probe may yield a diagnosis that is more indicative of disease severity than stenosis alone.

5.5 Therapeutics

Throughout this thesis, we have reiterated that the ferric state of iron can generate a highly pro-oxidant environment in a carotid plaque which may lead to increased lipid oxidation, increasing the inflammatory response as well as driving plaque progression through LDL oxidation. It would seem that removal of this inflammatory iron source would be beneficial to patient prognosis. We have shown that iron sequestration through chelation can inhibit the production of ROS in vitro. Others have shown that after the administration of these drugs, there is a measurable change in the MRI relaxometry parameters of various tissues (liver$^8$, myocardium$^9$).
Furthermore, application of these iron chelating drugs in animal models of disease have shown positive effects on plaque burden even in a study group without plaque hemorrhage\textsuperscript{10}. This suggests that there is a pool of free iron in the circulation that can be removed to improve plaque prognosis.

We hypothesize that in patients who have MR detected IPH in carotid artery plaques, treatment using iron chelation therapy will reduce MR relaxometry parameters (specifically R1). Sequestration of intraplaque iron in these patients will improve patient prognosis.

Patients presenting at the myelodysplastic clinic who have been scheduled to undergo iron chelation therapy for iron overload may be an ideal pilot group to study this therapeutic option. This study has already been submitted and approved by the Sunnybrook Research ethics board. Patients would be recruited and screened using Doppler ultrasound. Those presenting with a moderate to high degree of carotid artery stenosis (>50\%) would be included in the study. This inclusion criterion is to increase the chances of including a patient with MRIPH as incidence appears to increase with increasing stenosis.

All patients included will receive a baseline MRI to determine if IPH is present, and MRI relaxometry parameters will be acquired in those presenting with IPH. $T_1$ quantification, either through the Look-Locker methods as outlined in this thesis, or alternatively, techniques we are developing in the lab\textsuperscript{11} will be used to generate $T_1$ maps of the carotid artery along with the plaque haemorrhage. Additionally, plaque volume measurements would be calculated from acquiring a 3D volumetric MRI dataset. Furthermore, plaque enhancement after the injection of contrast agent gadofosveset, which we have shown in animal models to be correlated with macrophage infiltrate and neovascularisation\textsuperscript{12} will be calculated. A control group of patients not receiving iron chelation therapy but with previously identified MRIPH positive plaques would be used to observe the natural evolution of MR positive plaque. Patients would be re-imaged at 3, 6 and 12 months after the commencement of the study. Mean plaque volume, plaque volume change, IPH volume changes as well as quantitative measures of plaque $T_1$ will
be calculated. These numbers are not nearly large enough to show significance, however will serve as useful pilot data to determine sample size for a larger interventional study.

To test the second hypothesis patients would be followed out to the end of 3 years and contacted via telephone interview for signs of cerebrovascular symptoms.

5.5.1 Evaluating the use of Alternative Iron Chelation Agents

While the proven efficacy of DFO and similar chelators is attractive, the side effects and the route of administration of these agents may make this drug unfeasible for widespread use. Along with clinically proven chelation agents, other compounds will be tested in vitro in parallel with the patient trial described above. One agent of particular interest is the natural anti oxidant alpha lipoic acid (ALA). This compound has been used extensively in the naturopathic treatment of diabetes to treat diabetic retinopathy. Furthermore, scientific studies have shown it to be a useful antioxidant through reducing many oxidised materials\(^\text{13}\). Additionally, it has been shown to have some limited abilities to chelate iron byproducts\(^\text{14}\). While other antioxidant compounds such as vitamin C and E have had mixed results when applied in large clinical trials\(^\text{15}\), these may have a more focused effect when applied to a specific patient population previously screened to have a higher risk of events owing to a highly pro-oxidant environment such as those patients identified as having MR detected IPH. Our preliminary in vitro data suggests that ALA has approximately the same abilities to inhibit iron mediated lipid oxidation as does DFO.

A study that follows from this data is a specific targeting of patients who present with IPH to take a course of ALA. Currently, those patients who have less than what is classified as significant stenosis (<70%) and are asymptomatic so do not qualify for surgical intervention. The risks of surgery outweigh the benefits for these patients; however many of our outcome studies support the idea that an intervention may be required. These patients could be recruited into a randomised study that would assign one arm to the intervention whilst the other arm received placebo. Study design and
outcomes would be very similar to those detailed in the previous section looking at the effects of DFO, with measurements including decreases in haemorrhage volume, and plaque burden. Outcomes studies again for these trials would need to be based on a significantly larger volumes of these patients.
Concluding Remarks

Intraplaque haemorrhage may be an important marker of patient outcome. In this thesis, some in vitro evidence was shown supporting the clinical observation that MR detected IPH seems to predict patient outcome. Furthermore, we have demonstrated that there is a marked difference in how different protective proteins interact with the MR signal. However, the “cure for stroke” is probably neither simply detecting IPH nor determining the Hp phenotype of a patient. Most of our clinical IPH studies have only been done in males, and embolic stroke from such varied sources such as myocardial emboli have not been discussed in this work. The various avenues will continue to confound

However, the groundwork has been laid that identifies intraplaque ferric iron as both an imaging and therapeutic target in the prevention of embolic stroke from carotid atherosclerosis.

The time is now ripe for another NASCET type trial – however in this case testing the target of IPH. While the techniques currently implemented in MRI will continue to provide a grounding for any future knowledge gained, devices that bring this detection technology into the wider more accessible clinics of the developing world will enable a more accurate prognosis of future embolic stroke potential. Detecting plaque constituents, primarily haemorrhage, and not stenosis will then be the defining role of imaging. Looking beyond the lumen will have made all the difference.
5.6 References for Chapter 5


