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N.B.: When citing this work, cite the original article.

Original Publication:

Håkan Gustafsson, Martin Hallbeck, M. Norell, Mikael Lindgren, Maria Engström, Anders Rosén and Helene Zachrisson, Fe(III) distribution varies substantially within and between atherosclerotic plaques, 2014, Magnetic Resonance in Medicine, (2), 71, 885-892.

<http://dx.doi.org/10.1002/mrm.24687>

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Postprint available at: Linköping University Electronic Press

<http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-98001>

Fe(III) distribution varies substantially within and between atherosclerotic plaques

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Fax: Not available

Word count of body (not including the title page, abstract, figure captions, tables, table captions, or references): 4108 words.

Word count of abstract: 199 words.

Total number of figures and tables: 5 figures and 3 tables.

Objective: Vulnerable atherosclerotic plaques are structurally weak and prone to rupture, presumably due to local oxidative stress. Redox active iron is linked to oxidative stress and the aim of this study was to investigate the distribution of Fe(III) in carotid plaques and its relation to vulnerability for rupture.

Methods: Atherosclerotic plaques from ten patients (3 asymptomatic and 7 symptomatic) were investigated. Plaque vulnerability was classified using ultrasound and immunohistochemistry and correlated to Fe(III) measured by electron paramagnetic resonance spectroscopy.

Results: Large intra-plaque Fe(III) variations were found. Plaques from symptomatic patients had a higher Fe(III) concentration as compared to asymptomatic plaques (0.36 ± 0.21 vs. 0.06 ± 0.04 nmol Fe(III)/mg tissue, $P < 0.05$, in sections adjoining narrowest part of the plaques). All but one plaque from symptomatic patients showed signs of cap rupture. No plaque from asymptomatic patients showed signs of cap rupture. There was a significant increase in cap macrophages in plaques from symptomatic patients compared to asymptomatic patients (31 ± 11 % vs. 2.3 ± 2.3 %, $P < 0.01$).

Conclusion: Fe(III) distribution varies substantially within atherosclerotic plaques. Plaques from symptomatic patients had significantly higher concentrations of Fe(III), signs of cap rupture and increased cap macrophage activity.

Keywords

Atherosclerosis, oxidative stress, reactive oxygen species, iron, electron paramagnetic resonance (EPR).

Introduction

Atherosclerotic plaques vulnerable for rupture are characterised by a thin fibrous cap [1], a large lipid pool and a high number of inflammatory cells such as macrophages-derived foam cells [2-4]. Vulnerable plaques are also structurally weaker [5] and therefore more likely to rupture in response to physical forces [6]. Oxidative stress, i.e. imbalance between physiological oxidative defence mechanisms and reactive oxygen species (ROS), causes biological damage [7-9]. A weak fibrous cap in vulnerable plaques may be a result of oxidative damage by high local concentrations of ROS [9] produced by macrophage derived foam cells. It has also been demonstrated that high local concentration of ROS leads to increased activity of vascular matrix metalloproteinase (MMP) that further degrades the fibrous cap [6, 10].

Atherosclerotic plaques in the carotid arteries is a common source of thromboembolism and subsequent risk of stroke. Carotid endarterectomy (CEA) guidelines in symptomatic carotid artery stenosis are based on European Carotid Surgery Trial (ECST) and North American Symptomatic Carotid Endarterectomy Trial (NASCET) criteria with 70% or greater stenosis [11]. Also asymptomatic patients with a high grade internal carotid artery stenosis may benefit from carotid endarterectomy as demonstrated in the asymptomatic carotid atherosclerosis study (ACAS) [12] and the asymptomatic carotid surgery trial (ACST) [13]. Although ultrasonic duplex imaging of the carotid artery is routinely used in the clinic to assess the level of stenosis and blood flow, the value of ultrasonic duplex imaging for assessment of plaque composition and plaque vulnerability for rupture remains limited [14]. Previous results indicate that the ultrasound technique could provide useful information both for the evaluation of patient risk and for guidance of therapeutic decisions and monitoring. Contradictory results, however, make further investigations necessary. Some of these are

aimed at the description of plaque surface (the cap) and the subcapsular area with possible necrotic zones including macrophage activity.

A number of studies suggest that iron may play a key role in the pathogenesis of atherosclerosis [15-23]. Redox-active iron is known to act as a catalyst in the production of strong oxidative ROS (Fenton and Haber-Weiss reactions) [24-25] and it has been hypothesised [20] that elevated levels of iron *in vivo* lead to increased oxidative stress. Recently Raman et al. [19] used magnetic resonance imaging (MRI) to quantify iron content in carotid atherosclerotic plaques *in vivo* in human patients using standard methods for MRI tissue iron assessment [26-28] and showed that MRI can be used to distinguish symptom producing plaques from asymptomatic plaques. It is of interest to further study the relationship between iron levels in atherosclerotic plaques and vulnerability for rupture, especially since it has already been established that MRI can be used for assessment of other risk factors for rupture such as microemboli, cap-rupture, intra-plaque haemorrhage, plaque neovasculature and inflammation [29-30].

Various techniques have been used to study iron in biological systems [31]. Electron paramagnetic resonance (EPR) spectroscopy (equivalent to electron spin resonance (ESR) spectroscopy) offers a non-destructive method for studies of paramagnetic iron species to obtain information on e.g., the iron oxidation state and the type of iron-ligand bonding [32]. EPR spectroscopy can in addition be used for absolute quantifications of high spin ferric Fe(III) ions in frozen samples in the micro-molar range [31]. The EPR spectra of frozen human carotid atherosclerotic plaques consist to various degrees of signals assigned to haem iron, non-haem high-spin iron (e.g. iron bond in transferrin) and low-spin iron [16-17, 19, 32-33]. Although some states of Fe(II) are paramagnetic, an EPR signal is not typically observed

for Fe(II) due to very strong spin-orbit coupling [32-33] giving rise to substantial line-broadening.

The high reactivity of some of the ROS (e.g. hydroxyl radicals) limits the distance between the chemical origin and the point for oxidative damage. We hypothesized that vulnerability for rupture of carotid atherosclerotic plaques is better correlated to intra-plaque iron distribution and therefore to the localization of oxidative stress, rather than to a high mean iron concentration in the plaque. In the present work we therefore quantified the distribution of iron both in plaques from symptomatic and asymptomatic patients with the aim of exploring the relationship between elevated local levels of redox active iron and plaque vulnerability for rupture as classified by *in vivo* and *ex vivo* ultrasound and compared to histopathological examination.

Materials and methods

Patient data

Ten patients undergoing carotid endarterectomy (CEA) were prospectively and consecutively enrolled for study participation. Seven patients had symptomatic ICA (internal carotid artery) stenosis (amaurosis fugax, n = 2; minor stroke n = 4; transient ischaemic attack (TIA), n = 1) and 3 patients were asymptomatic. The symptomatic patient group was represented by 5 males (mean age 75 years (y); range 60-83 y) and 2 women (mean age 58 y; range 53-63 y). The asymptomatic patient group was represented by 2 males (mean age 69 y; range 55-83 y) and one woman (age 68 y). The study was approved by the Local Ethical Review Board in Linköping, Sweden. The plaques were surgically removed and immediately fixed in 40 % formaldehyde.

Duplex ultrasound in vivo

Preoperative duplex ultrasound of the neck vessels was performed within one week before carotid endarterectomy (CEA) in the seven symptomatic patients and one month before CEA in the three asymptomatic patients. Ultrasound B-mode imaging of the neck vessels was performed by a high frequency ultrasound Acuson S2000 scanner (Siemens, Mountain View, CA) equipped with a 9 MHz -18 MHz transducer. All conventional ultrasound measurements were performed at an angle of 60 degrees at maximum between the transducer and the blood flow direction and saved for further offline analysis using computerized image analysis software; Adobe Photoshop CS5 (Adobe systems, Mountain View, CA) The degree of ICA stenosis was measured by peak systolic flow velocities (PSV) to assess the degree of stenosis (ECST method) [34]. Plaque composition was objectively assigned as fibrous, fibro-atheromatous or atheromatous and the caps were classified as smooth, irregular, or ulcerated.

Duplex ultrasound ex vivo

All ten carotid plaques were moulded into gelatine in special Plexiglas boxes (15 cm × 8 cm × 5 cm) directly after CEA to allow for *ex vivo* ultrasound examination and subsequent quantification of Fe(III) using EPR spectroscopy and immunohistology. The moulding was performed in two steps: First 18.2 g of gelatine leafs (gelatine from animal origin, Dr. Oetker, Mölndal, Sweden) were dissolved in 3.5 dl water in a beaker using a magnetic stirrer/heater until completely dissolved. The solution was then cooled down to 37 °C by placing the beaker in a water bath and then poured into the Plexiglas box to a height of 3 cm. The gelatine was then allowed to solidify by placing the Plexiglas box into a refrigerator (8° C). After the gelatine had solidified a top layer of gelatine solution was prepared as described above and poured into the Plexiglas box to fill it up completely. Using a pair of tweezers the carotid plaque was placed at the bottom of the top layer of gelatine (2 cm from the surface of

the top layer). Care was taken to remove all air bubbles from the surface of the plaque, within the lumen of the plaque, at the surface of the bottom layer and at the surface of the top layer of gelatine. The top layer with plaque was then allowed to solidify by placing the box into the refrigerator again. Ultrasound B-mode imaging of the *ex vivo* plaques were performed using the same equipment as used for *in vivo* ultrasound. All carotid specimens underwent ultrasound examinations in both 2D and 3D to carefully determine the positions of the most constricted part of the plaque lumen which were documented with marks on a photograph of the plaque in its box.

Histopathology

From each of the ten plaques a 4 mm thick slice from the most constricted part of the vessel as determined by ultrasound was removed. This section was formalin fixed and paraffin embedded using standard methods for subsequent histopathological evaluation. The plaque parts distal and proximal to this centre slice were stored for subsequent quantification of Fe(III) using EPR spectroscopy (described below). For histopathology 4 µm thick sections were sectioned. One of these was used for standard hematoxylin and eosine staining (H&E). Subsequent sections were used for immunohistochemical staining with anti-CD68 antibody (Ab) (PG-M1, Dako, 1:100), anti-CD163 Ab (10D6, Leica Novocastra, 1:100), anti-CD31 Ab (JC/70A, Dako, 1:50) and anti-SMA Ab (1A4, Dako, 1:300). Staining was performed by using IntelliPATH FLX (BioCare). All stained sections were subsequently digitalized (ScanScope AT Turbo, Aperio). All cases were analysed by an experienced pathologist (M.H.). Analysis of the digitalized sections was performed with ImageScope (Aperio), the H&E section were used as the basis for analysis from which cap thickness and the presence of cap rupture could be analysed. The percentage of foamy macrophages within the cap was calculated by measuring the areas containing lipid laden macrophages (asserted on H&E,

confirmed by positive CD68 and CD163 staining) and dividing this with the total area of the cap. Area measurements were done using the ImageScope. Presence of rupture was evaluated on the H&E section as was measurements of cap thickness, diameter of the lipid core and presence of calcification. Arbitrary values were given to the presence of macrophages outside of the plaque, microvascular proliferation in the cap (determined by CD31 and SMA labelling) and in other parts of the slide, cap thickness and hemosiderin content and presence of calcification. The observations from each slide were assigned numerical values based on the relative prominence: none = 0; minimal = 0 – 1; moderate = 1 – 2; extensive = 2 – 3. In some cases small parts of the section were damaged during sectioning procedure due to calcifications, but in the sections used, the damage was not significant enough to preclude evaluation. Two of the symptomatic case was too damaged to permit histopathological evaluation and had to be excluded from this part of the study.

The plaque composition was classified as atheromatous, fibroatheromatous or fibrous.

Presence of cap rupture was classified as “yes” or “no”.

Quantification of Fe(III)

All EPR measurements were performed using a Bruker E500 Eleksys X-Band EPR spectrometer equipped with a SHQE resonator at 150 K using a Bruker ER 4111 VT variable temperature unit. Spectrometer settings were: applied microwave power 20 mW, modulation frequency 100 kHz and modulation amplitude 0.5 mT, appropriate to avoid saturation and /or over modulation of the Fe(III) signal. For simple comparison of signal strength all spectra were recorded with a sweep width 160 mT, 1024 points, time constant 10 ms with 20 sweeps added together for each measurement. Samples were analysed in an 8 mm tube (precision bore suprasil tube 513A-1PP-7SUP) fitted in a special made Dewar (WG-821-TMR-SPECIAL) both from Wilmad-LabGlas (Vineland, NJ). No EPR signal could be detected for

the empty sample tube or phosphate buffered saline (PBS). Plaque parts distal and proximal to the section used for histology (see section above) for each plaque were cut transversally in sections (30 mg – 70 mg each). Each section was placed into a syringe and PBS was added to a total (sample + PBS) volume of 100 μ L to maintain the same sample volume. The syringe was frozen in liquid nitrogen, removed and warmed in the palms of the hands until a slightly thawed 100 μ L cylinder could be pressed out into the deep frozen sample tube, which was immediately placed in the EPR resonator. Signal intensity variation due to variations in microwave coupling and Q-value for all samples was minimised as the sample volumes were similar. The recorded EPR spectra were imported [35] into MATLAB (version R2011a, MathWorks, Inc.), baseline corrected and double integrated using an in-house developed MATLAB code. The EPR signal intensities were mass normalised. The quantity of Fe(III) in nmol Fe(III)/mg tissue could be calculated using calibration curves obtained from a series of repeated measurements on calibration samples (Fe(III)-deferoxamine 1:1 complex in PBS) in the range 0 mM Fe(III) (PBS only) to 2.00 mM Fe(III), typically in steps of 0.25 mM. The detection limit was approximately 0.01 nmol Fe(III)/mg sample.

Statistical analysis

All statistical analyses were performed using SPSS v.19 (IBM SPSS Statistics 19). Descriptive statistics are presented as mean $\pm$ standard deviation (SD) unless otherwise stated. Quantitative variables were analysed with independent samples Student t-tests and p values < 0.05 were considered statistically significant.

Results

Duplex ultrasound in vivo

The degree of ICA stenosis measured as peak systolic flow velocities (PSV) showed no statistical significance between the groups ($4.9 \text{ m/s} \pm 1.8 \text{ m/s}$ (symptomatic) vs. $3.4 \text{ m/s} \pm 0.2 \text{ m/s}$ (asymptomatic), ($P > 0.05$)). The degree of ICA stenosis in symptomatic patients were determined to be 80 % - 99 % ($n = 6$) and 70 % - 79 % ($n = 1$). The degree of ICA stenosis in asymptomatic patients were 80 % - 99 % ($n = 3$). Contralateral significant ICA disease was seen in 3 symptomatic patients (occlusion, $n = 1$ and significant ICA-stenosis 50 % -79 %, $n = 2$). The asymptomatic plaques were classified as fibrous ($n = 2$), fibro-atheromatous ($n = 1$). The symptomatic plaques were classified as fibrous ($n = 1$), fibro-atheromatous ($n = 2$) and atheromatous ($n = 4$). In the symptomatic patient group all patients had signs of irregular cap surface combined with ulceration in two plaques. In the asymptomatic patient group two plaques had signs of smooth plaque surface and one had signs of irregular plaque surface. Plaque compositions and cap classifications are given in Table 1.

Histopathology

In all but one symptomatic plaques rupture could be identified while none of the asymptomatic plaques showed signs of rupture. The proportion of the cap that contained foamy macrophages in the symptomatic plaques was significantly higher compared to asymptomatic plaques ($31 \pm 11 \%$ vs 2.3 ± 2.3 , $P < 0.01$). A tendency of higher hemosiderin score could be seen in the symptomatic plaques but this did not reach statistical significance (results not shown). The presence of macrophages outside of the plaque, microvascular proliferation in the cap and in other parts of the section, and presence of calcification did not reach statistical significance between the groups. A typical example of an asymptomatic plaque with intact cap and few macrophages is shown in Figure 1, panel A. A symptomatic plaque with rupture and extensive invasion of foamy macrophages within the cap is shown in panel B.

EPR spectroscopy

The EPR spectra of the slices from the 10 carotid atherosclerotic plaques consisted of haem iron signals, non-haem high-spin iron (e.g. iron in transferrin) and low-spin iron. A representative spectrum is shown in Figure 2 A with the low field signal from haem iron centred on $B_0 \approx 1200$ G ($g = 6$), non-haem high-spin iron (e.g. iron bond in transferrin) centred on $B_0 \approx 1600$ G ($g = 4.3$) and low-spin species centred on $B_0 \approx 3400$ G ($g = 2$). Figure 2 B shows a magnification of the $g = 6$ and $g = 4.3$ signals (g is a dimensionless quantity, which characterises the magnetic moment and gyromagnetic ratio of electrons and can be used to identify paramagnetic species in an EPR spectrum [32]).

Quantification of Fe(III)

Signal intensity analysis (of the peaks at $g = 4.3$) showed large intra-plaque variations in Fe(III) concentrations with values ranging from below detection limit (0.01 nmol Fe(III)/mg sample) up to 1.00 nmol Fe(III)/mg tissue within the same plaque. Data of all plaques are summarised in Table 1, Table 2, Table 3 and Figure 3. The mean Fe(III) concentration was significantly larger in symptomatic plaques (0.29 ± 0.08 nmol Fe(III)/mg sample) as compared to asymptomatic plaques (0.11 ± 0.09 nmol Fe(III)/mg sample) ($P < 0.05$) (Table 2). When only considering the two to three sections adjoining the narrowest part of the plaque lumen, the difference was (0.36 ± 0.21 Fe(III)/mg sample) as compared to asymptomatic plaques (0.06 ± 0.04 Fe(III)/mg sample) ($P < 0.05$) (Table 2). Table 1 shows the measured mean Fe(III) concentrations and maximum and minimum values for all plaques. Table 3 gives all the Fe(III) concentration in all sections for all plaques. Shaded areas and underlined numbers in Table 3 indicate sections adjoining the narrowest part of the plaques as determined with *ex vivo* ultrasound. The distribution of Fe(III) in one asymptomatic plaque

(plaque #1) and one symptomatic plaque (plaque #10) is shown in Figure 4 and Figure 5 respectively. The dashed lines in Figure 4 and Figure 5 indicate the narrowest part for these plaques.

Discussion

These data support two main conclusions: (1) Symptomatic patients had significantly higher concentrations of Fe(III) as quantified by EPR spectroscopy, as well as signs of cap rupture and increased cap macrophage activity, as compared to asymptomatic patients. (2) The intra-plaque distribution of Fe(III) in *ex vivo* atherosclerotic plaques is heterogeneous.

Intra-plaque haemorrhage and presence of neovascularization and have been correlated with the incidence of earlier cardiovascular events [36-37]. Visualization of neovascularization and haemorrhage can be performed noninvasively using imaging modalities such as contrast-enhanced ultrasound and MRI [38]. In this study we performed an *ex vivo* analysis using electron paramagnetic resonance (EPR) spectroscopy to assess data concerning differences in iron content as a sign of haemorrhage in clinical silent plaques as compared to plaques provoking neurological symptoms.

Stadler et al. [16] used both EPR spectroscopy and inductively coupled plasma mass spectroscopy (ICP-MS) to quantify iron in *ex vivo* carotid plaques. They found that carotid plaques that had been classified as “complex” or “calcified” contained statistically higher levels of Fe(III) as compared to healthy intima samples or plaques classified as “clean” and concluded that elevated levels of Fe(III) may be related to plaque instability. Raman et al. [19] found that T_2^* (an MRI relaxation parameter measured in ms) was significantly shorter in symptomatic patients as compared to asymptomatic patients, which indicated higher amounts of T_2^* shortening by iron aggregates in symptom producing atherosclerotic plaques.

However, Raman et al. [19] found, contradictory to Stadler et al. [16], lower levels of Fe(III) in plaques from symptomatic patients, as compared to asymptomatic patients.

In this study we included plaques both from symptomatic patients referred to ultrasound examination because of neurological symptoms (TIA, minor stroke and amaurosis fugax) as well as asymptomatic patients (i.e. carotid plaques without neurological symptoms).

Various techniques such as ultrasound, magnetic resonance imaging (MRI), dual energy CT (DECT) and positron emission tomography (PET) are available for visualization of carotid plaques. MRI has been shown to be useful for identification of risk factors for rupture such as vascular wall inflammation, fibrous cap, lipid rich necrotic core and neovascularization [29-30]. However, despite recent developments of conventional imaging modalities it is still a clinical challenge to predict the vulnerability for plaque rupture and subsequent embolic stroke [14].

Oxidative stress is a causative factor for the pathogenesis of vascular disease [9] and iron may act as a catalyst for the generation of ROS during its cycling between oxidation states Fe(III) and Fe(II) by means of Fenton and Haber-Weiss reactions [24, 32]. Iron could therefore be central in the pathogenesis of atherosclerosis. It has even been postulated that the gender differences seen in death from heart disease depends on the gender differences in body iron stores [15, 20].

Almost all iron in the body is tightly bound by proteins in redox inactive forms such as serum ferritin [18, 21] and can thus not act as a catalyst for ROS. However, it is known that iron can be released from ferritin by several pathological processes [25] and thus ferritin-dependent oxidative damage can be induced by pathological leakage of iron from e.g. ferritin to form “catalytic” or “labile” iron [23], in which the iron is bound by low molecular weight ligands

like organic acids and nucleotides [18].

All our ten carotid atherosclerotic plaques gave EPR spectra that to various degrees consisted of haem iron signals, non-haem high-spin iron (e.g. iron in transferrin) and low-spin iron (Figure 2). We choose to quantify the amount of non-haem high-spin iron (e.g. iron in transferrin) in sections of each plaque using quantitative EPR spectroscopy at 150 K using signal intensity comparisons with calibration samples (Fe(III)-deferoxamine 1:1 complex in PBS) with known concentrations. Our method for quantitative EPR of Fe(III) with measurements on frozen 100 μ L discs in a 8 mm tube fitted in a special made Dewar allowed for high measurement accuracy and precision as estimated from a series of repeated measurements on both calibration samples and carotid plaques. Our results (Table 2 and Figure 3) are in agreement of the findings by Stadler et al. [16] with higher mean non-haem high-spin iron ($g = 4.3$) concentrations in atherosclerotic plaques from symptomatic patients. We also found that the intra-plaque distributions of non-haem high-spin iron (e.g. iron in transferrin) are very heterogeneously distributed in carotid atherosclerotic plaques as exemplified by Figure 4 and Figure 5.

The histopathological examination revealed that the symptomatic plaques had significant higher presence of macrophages and presence of rupture in all but one case. These differences confirmed the symptomatic plaques as vulnerable. Other classical signs of vulnerable plaques such as larger lipid core, thinner fibrous cap or microvascular proliferation [39] were not found to differ in the present material. Nevertheless the sign of cap rupture in combination with increased macrophage activity that we found in this study could be a critical sign of vulnerability. There could be several reasons for this discrepancy of vulnerability signs and the main one being the small number of cases in the present study. We

must also consider the possibility that differences exist between the mostly described coronary vessel plaques and those in the carotids. It can be argued that an increase in iron could be secondary to neovascularization, but the histopathological examination did not support this assumption. However, a tendency to higher hemosiderin content in the symptomatic, vulnerable plaques was observed. This could be an indirect sign of increased leakiness in less well endothelialized vessels as present during neovascularization.

Whether differences in plaque structure alone are enough to distinguish between symptomatic and clinically silent lesions has yet to be established [34]. It has been reported that ultrasonic echolucent carotid plaques predict future strokes [40], but the cap surface might be the best ultrasound predictor to assess the vulnerability of the plaque as shown to be ruptured in the symptomatic plaques.

Obviously, as expected, other risk factors, in addition to the peak value of Fe(III) concentration, are important for plaque vulnerability for rupture. Our hypothesis that vulnerability for rupture of carotid atherosclerotic plaques is correlated to intra-plaque iron distribution is to some extent but not fully supported by the tendency of larger difference in mean iron concentration between the groups when only considering the position determined as rupture prone (most narrow) using ultrasound (Table 2). While the number of plaques ($n = 10$) has to be increased in further studies, the results indicate that the distribution of iron in atherosclerotic plaques can be associated with local vulnerability for rupture. We believe that dedicated MRI studies are needed in the future to establish if the mean iron concentration alone can be used to predict plaque vulnerability for rupture, or if the mean iron concentration rather should be evaluated together with MRI observations of other risk factors such as lipid rich necrotic core, vascular wall inflammation et cetera. Our observation that the Fe(III)

distribution varies substantially within atherosclerotic plaques could possible point towards that the latter combined approach is needed. Fe(III) concentrations up to 0.92 nmol Fe(III)/mg tissue in one tissue section were observed in one plaque from an asymptomatic patients (Table 3, section 6 of plaque #3). Interestingly ultrasound showed signs of irregular cap surface in this plaque indicating high risk of embolisation compared to the other two asymptomatic plaques with signs of smooth cap. Plaque #3 therefore mimicked the symptomatic plaques concerning cap surface whereas all symptomatic plaques had signs of irregular cap and / or ulceration. The vulnerability of the plaque is especially important to assess in asymptomatic patients before the onset of neurological symptoms.

One could argue that the highest risk of embolisation is supposed to occur when the cap of the plaque is broken due to high concentration of macrophages in the cap. We therefore included macrophage activity as well as description of the cap in the first place in this study and did not include oxidative epitopes which however could be an important topic for further studies.

Conclusion: The results of this study indicate that Fe(III) distribution varies substantially within atherosclerotic plaques. Plaques from symptomatic patients had significantly higher concentrations of Fe(III), as well as signs of cap rupture and increased cap macrophage activity.

Acknowledgments

H. Gustafsson acknowledges The Swedish Research Council (diariennr 2009-5430) for financial support and Stiftelsen Lars Hiertas Minne for travel grant (FO2009-0345). M. Engström acknowledges the Medical Research Council of Southeast Sweden (FORSS) for

financial support. M. Lindgren thanks Linköping University for the tenure as distinguished guest professor. We thank B. Frånlund and L. Palmqvist for histopathological preparations.

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Figure 1. Panel A shows a typical example of an asymptomatic plaque with intact cap and little macrophages. Panel B shows a symptomatic plaque with rupture (arrowheads) and extensive invasion of foamy macrophages (arrows) within the cap. Scale bar = 200 μm .

Figure 2. Panel A shows a representative EPR spectrum from a frozen human carotid atherosclerotic plaque (one section obtained from plaque # 4, symptomatic patient) with a low field signal from haem iron centred on $B_0 \approx 1200$ G ($g = 6$), non-haem high-spin iron (e.g. iron bond in transferrin) centred on $B_0 \approx 1600$ G ($g = 4.3$) and low-spin species centred on $B_0 \approx 3400$ G ($g = 2$). Panel B shows a magnification of the $g = 6$ and $g = 4.3$ signals.

Figure 3. Mean Fe(III) concentration in the two - three sections adjoining the narrowest part of the lumen (nmol Fe(III) /mg tissue). Plaques 1 - 3 from asymptomatic patients. Plaques 4 - 10 from symptomatic patients. Significantly higher mean Fe(III) in sections from symptomatic patients as compared to sections from asymptomatic patients ($P < 0.05$).

Figure 4. Experimental results from EPR spectroscopy of asymptomatic plaque number 1. Dashed line indicates the narrowest part of the lumen as indicated by ex vivo ultrasound.

Figure 5. Experimental results from EPR spectroscopy of symptomatic plaque number 10.

Dashed line indicates the narrowest part of the lumen as indicated by ex vivo ultrasound.

Table 1. Clinical classification, plaque composition, cap surface and mean, maximum and minimum Fe(III) concentration for all plaques.

plaque #	clinical classification	Plaque composition (fibrous, fibro- atheromatous or atheromatous)	cap surface (smooth, irregular or ulcerated)	mean Fe(III) concentration in all tissue sections (nmol Fe(III) /mg tissue)	mean Fe(III) concentration in tissue sections adjoining narrowest part (nmol Fe(III) /mg tissue)	Maximum intra- plaque Fe(III) concentration (nmol Fe(III) /mg tissue)	Minimum intra- plaque Fe(III) concentration (nmol Fe(III) /mg tissue)
#1	asymptomatic	fibrous	smooth	0.08	0.07	0.25	< 0.01
#2	asymptomatic	fibro-atheromatous	smooth	0.03	0.02	0.09	< 0.01
#3	asymptomatic	fibrous	irregular	0.21	0.10	0.92	< 0.01
#4	symptomatic	fibro-atheromatous	irregular	0.34	0.60	0.62	< 0.01
#5	symptomatic	fibrous	irregular	0.15	0.12	0.37	< 0.01
#6	symptomatic	atheromatous	irregular	0.26	0.31	0.38	0.16
#7	symptomatic	fibro-atheromatous	ulcerated	0.27	0.36	0.54	0.11
#8	symptomatic	atheromatous	irregular	0.28	0.23	0.76	0.04
#9	symptomatic	atheromatous	irregular	0.31	0.24	0.70	< 0.01
#10	symptomatic	atheromatous	ulcerated	0.43	0.69	1.00	< 0.01

Table 2. Mean Fe(III) concentration for all plaques from symptomatic (n=7) and asymptomatic patients (n =3). Values are given as mean value $\pm$ standard deviation (k=1).

clinical classification	mean Fe(III) concentration in all sections	mean Fe(III) concentration in the sections
		adjoining narrowest part
	(nmol Fe(III) /mg tissue)	(nmol Fe(III) /mg tissue)
asymptomatic	0.11 $\pm$ 0.09	0.06 $\pm$ 0.04
symptomatic	0.29 $\pm$ 0.08*	0.36 $\pm$ 0.21*

*Significantly higher versus plaques from asymptomatic patients ($P < 0.05$).

Table 3. Fe(III) in all tissue sections for all plaques. Shaded areas indicate tissue sections adjoining narrowest part of the plaques as determined with ex vivo ultrasound.

nmol Fe(III) /mg tissue	plaque #1	plaque #2	plaque #3	plaque #4	plaque #5	plaque #6	plaque #7	plaque #8	plaque #9	plaque #10
section 1	< 0.01	< 0.01	0.50	< 0.01	<u>0.07</u>	0.27	0.13	<u>0.06</u>	< 0.01	< 0.01
section 2	0.02	0.09	<u>0.17</u>	0.15	<u>0.16</u>	0.16	0.22	<u>0.41</u>	< 0.01	0.05
section 3	< 0.01	<u>0.04</u>	<u>0.02</u>	<u>0.59</u>	0.15	0.26	0.31	0.76	0.29	0.15
section 4	0.15	< 0.01	< 0.01	<u>0.62</u>	< 0.01	<u>0.24</u>	0.11	0.64	0.39	0.25
section 5	0.07	0.06	0.17	0.19	0.37	<u>0.38</u>	<u>0.18</u>	0.14	0.30	0.42
section 6	<u>0.05</u>	< 0.01	0.92	0.27	0.28		<u>0.54</u>	0.13	0.24	0.42
section 7	<u>0.02</u>	< 0.01	< 0.01	0.35	< 0.01		0.42	0.14	< 0.01	<u>0.68</u>
section 8	<u>0.15</u>	0.03	0.11	0.53				0.17	<u>0.48</u>	<u>0.70</u>
section 9	0.14		0.12					0.27	0.70	0.64
section 10	0.25		0.11					0.04	0.67	0.55
section 11	0.04									0.39
section 12	0.03									0.44
section 13										0.46
section 14										1.00
section 15										0.23