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**Neopterin and 7,8-dihydroneopterin are generated within atherosclerotic plaques**

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**Abstract:** Plasma neopterin correlates with the level of cardiovascular disease. Neopterin is the oxidation product of 7,8-dihydroneopterin, which is released by γ-interferon-stimulated macrophages. 7,8-Dihydroneopterin is a potent antioxidant, which inhibits lipid oxidation, macrophage cell death and scavenger receptor CD36 expression. The concentration of neopterin within atherosclerotic plaques was measured in tissue removed from carotid and femoral arteries. The excised plaques were cut into 3-mm-thick sections, and each section was analysed for neopterin, total neopterin, cholesterol, lipid peroxides, α-tocopherol and protein-bound 3,4-dihydroxyphenylalanine. Selected plaques were placed in tissue culture, and the media was analysed for 7,8-dihydroneopterin and neopterin release. Total neopterin levels ranged from 14 to 18.8 nmol/g of tissue. Large ranges of values were seen both within the same plaque and between plaques. No correlation between neopterin and any of the other analytes was observed, nor was there any significant trend in levels along the length of the plaques. γ-Interferon stimulation of cultured plaque generated total neopterin concentrations from 1 to 4 nmol/(g 24 h). The level of 7,8-dihydroneopterin generated within the plaque was within the range that inhibits lipid oxidation. The data show that atherosclerotic plaques are extremely dynamic in biochemistry and are the likely source of the plasma 7,8-dihydroneopterin and neopterin.

**Keywords:** 7,8-dihydroneopterin; atherosclerosis; lipid peroxide; neopterin; plaque; protein bound DOPA; tocopherol.

**Introduction**

Atherosclerosis is a chronic inflammatory disease characterised by intimal thickening and deposition of macrophages in the artery wall. The inflammatory nature of the disease is highlighted by the correlation between plasma neopterin levels and increasing severity of cardiovascular disease [1–4]. Neopterin is a highly fluorescent pterin compound, whose measurement in urine and plasma has been extensively used as a marker of inflammation in a wide number of inflammatory conditions including vascular disease [5, 6]. During inflammation, γ-interferon released by T cells stimulates the enzymatic breakdown of guanosine-5’-triphosphate to 7,8-dihydroneopterin in human macrophages. Neopterin is the oxidation product of 7,8-dihydroneopterin, both of which are released into the tissues and blood from γ-interferon-activated macrophages.

Levels of serum neopterin have been correlated with atherosclerotic plaque destabilisation [7], plaque vulnerability [2], necrotic core volume in coronary plaques [8], and development of complex carotid plaque morphology [9], demonstrating the central role of macrophage inflammatory activity in plaque development.

The function of neopterin and 7,8-dihydroneopterin release by the macrophage has been less clear. Neopterin causes significant cellular stress at elevated concentrations [10–12], while 7,8-dihydroneopterin has been shown to be a potent antioxidant capable of inhibiting copper, peroxyl radical and cell-mediated oxidation of low-density lipoprotein (LDL) [13–15]. Macrophage uptake of oxidised LDL (oxLDL) is decreased by 7,8-dihydroneopterin-mediated down-regulation of the CD36 scavenger receptor [16]. Macrophage necrosis by HOCl, oxLDL and peroxyl radicals is also inhibited by 7,8-dihydroneopterin [16–19]. HOCl reacts extremely rapidly with 7,8-dihydroneopterin to generate neopterin [20, 21], suggesting that a significant amount of
the neopterin measured in serum and urine may be from the scavenging of HOCl released by neutrophils and macrophages during inflammation. These in vitro studies have led to the proposal that 7,8-dihydroneopterin is generated by macrophages to protect these cells from oxidants generated during inflammation [6, 13, 22, 23].

Although there has been a large volume of clinical studies on neopterin and, to a much lesser extent, 7,8-dihydroneopterin, few have looked directly at the actual sites and potential source of macrophage-generated 7,8-dihydroneopterin and neopterin. We have previously shown that human pus contains neopterin at concentrations ten- to a hundred-fold higher than human plasma, supporting the view that the source of 7,8-dihydroneopterin and neopterin in plasma is the actual site of inflammation [24]. Immune staining of carotid plaque has shown regions with high levels of neopterin associated with macrophages [7, 9]. Our previous analysis of atherosclerotic plaques removed from carotid arteries showed neopterin concentrations ranging from 0.72 to 2.34 μmol/L within the whole plaque [15]. Within one individual plaque that we sectioned, we reported large variations in neopterin concentration ranging from 0.4 to 2.2 nmol/g of wet tissue [6]. Based on these limited data, there appears to be a large range of neopterin concentrations both between plaques and within an individual plaque. As 7,8-dihydroneopterin is oxidised to neopterin, measurement of both compounds along the length of multiple plaques may reflect the dynamics of both macrophage activation and the oxidative environment across the various regions of plaque experience.

In this study, we examined a total of 29 surgically removed plaques from Christchurch Hospital, which were then cut into approximately 3-mm-thick sections along the longitudinal axis. The concentration of neopterin, cholesterol, oxidised lipids [thiobarbituric acid reactive substances (TBARS)], α-tocopherol (vitamin E) and protein-bound 3,4-dihydroxyphenylalanine (DOPA) was determined in the individual sections to generate a profile of the various analytes along the length of the plaques. Protein-bound DOPA was analysed as it is a product of both hydroxyl radical- [25] and HOCl [26]-mediated protein oxidation and has been previously identified in atherosclerotic plaques [27].

Materials and methods

All solutions and reagents were prepared using Nanopure water prepared using the Milli Q™ Millipore system. Chemicals and reagents were supplied from either Thermo Fisher Scientific, Auckland, New Zealand, Sigma Chemical Company, Sydney, Australia, or BDH Chemicals, Auckland, New Zealand Limited, unless otherwise stated. All tissue culture plasticware was supplied from Greiner Bio-one (Germany) through Raylab, Auckland, New Zealand Ltd. All high-performance liquid chromatography (HPLC) analysis was carried out using either a Shimadzu SIII0A or a SIl20A system equipped with a temperature control autosampler, a column oven and an RF-10 Axl spectrofluorometric detector. Peak areas were integrated using the Shimadzu LabSolutions™ software package (v1.22 SP1), and concentrations were calculated by comparison to the peak area of standards.

Carotid and femoral atherosclerotic plaques were surgically removed from consented patients undergoing carotid or femoral endarterectomy at Christchurch Hospital. Ethics approval for the study was granted by the Upper South A Regional Ethics Committee (ethics reference no. CTY/01/04/036).

Endarterectomy surgery involves physically peeling the intima plaque material off the artery, leaving the arterial wall structure and muscle layers intact. The material collected therefore did not contain the smooth muscle tissue of the artery. Thus the biochemical analysis was carried out on the pathological tissue rather than on the whole artery. This means that “healthy” arteries taken in other procedures are not true control material and were therefore not examined.

The plaque specimens were transported to the laboratory on ice and snap frozen in liquid nitrogen within 1 h of surgery. The plaque specimens were stored at –80°C for up to 6 months before analysis. Plaques were numbered from 1 to 35 sequentially as they were collected. Not all plaques supplied from surgery were intact, and damaged plaques were not used for biochemical analysis.

Plaques used for culturing were transported to the laboratory on ice where they were immediately cut into approximately 3-mm-thick rings and placed into individual wells in a 12-well tissue culture plate containing 1 mL of RPMI1640 supplemented with penicillin (100 U/mL), 100 μg/mL streptomycin and 10% human serum prepared from unlinked whole blood supplied by NZ Blood Service (Christchurch, New Zealand) (ethics approval no. CTY/98/07/069). Plaque lactate release was measured using the lactate assay kit from Roche Diagnostics (Indianapolis, IN, USA).

For biochemical analysis, selected plaques, while frozen, were cut into approximately 3-mm-thick rings using a scalpel along the longitudinal axis beginning from the proximal broad end to the distal narrow end in the direction of the blood flow. The individual sections were photographed and weighed before being ground to a fine powder using a pestle and mortar under liquid nitrogen. The resultant powder from each section was placed into 3.5 mL of water containing 1 mg/mL of EDTA and 200 μg/mL of the antioxidant butylhydroxytoluene.

Neopterin and total neopterin (neopterin plus 7,8-dihydroneopterin) were measured by reverse-phase HPLC analysis with fluorescence detection as previously described [28]. During the study, improvements in HPLC analysis from plaque number 19 onwards allowed measurement of both neopterin and the unoxidised form of 7,8-dihydroneopterin. 7,8-Dihydroneopterin measurement at the wavelength found within plasma and tissues (<50 nmol/L) requires oxidation of 7,8-dihydroneopterin to the highly fluorescent neopterin. The amount of neopterin measured after iodide oxidation is traditionally expressed as total neopterin as it is a measure of the neopterin plus 7,8-dihydroneopterin oxidised to neopterin [28].

Cholesterol content was determined using the Chol MPR 2 kit supplied by Roche Chemicals (New Zealand). Lipid oxidation products were measured as TBARS by reverse-phase HPLC with
fluorescence detection [24, 29, 30]. α-Tocopherol (vitamin E) was determined by hexane extraction of the homogenates, drying under nitrogen and analysis of methanol-solubilised hexane residue by reverse-phase HPLC with fluorescence detection [13]. Protein-bound DOPA was determined by gas-phase acid hydrolysis of acetone-delimitted plaque homogenates before HPLC analysis as previously described [25, 26].

The statistical analysis was performed using Prism (versions 4.0 and 5.0, GraphPad Software, La Jolla, CA, USA) and Statistica (version 9.0, StatSoft, Inc., USA). Correlation analysis was performed using R version 3.1. All data are represented as mean ± standard error of the mean of triplicate analysis of a homogenised sample.

**Results**

Thirty-five plaques in total were collected from surgery, of which 29 were intact, allowing sectional analysis of the concentrations of the selected analytes. Patient ages ranged from 60 to 86 years. Eight of the plaques collected were from the femoral artery, with the remainder from the carotid artery bifurcation (Table 1). Patients had been referred for surgery after presenting with a range of symptoms including transitory ischemic attacks, stroke and amaurosis fugax. Levels of stenosis determined by ultrasonound ranged from 50% to 95%. Patients were on a range of medications, but the majority were being treated with a combination of statins, antithrombotics and angiotensin-converting enzyme inhibitors.

The excised plaques varied greatly in their size, shape and composition. Extensive areas of calcification could be clearly seen in many of the cut sections along with regions of lipid deposition. Many plaques showed substantial levels of thrombosis through ulceration of the intima lining. Levels of calcification appeared

<table>
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<th>Total mass, g</th>
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Table 1: Summary of analysed plaques.

Plaques were collected and numbered sequentially on collection. Only plaques subject to analysis are listed in the table. Some plaques were recovered from surgery in multiple pieces and were not suitable for analysis. The general appearance of the plaques was defined as either having a bifurcation or being a single tube shape. Plaques were further defined as whether they were heavy calcified, contained significant thrombus regions or were mainly thickened fibrous intima. The level of stenosis was defined by Doppler ultrasound as part of the patient pre-surgical diagnosis but was not carried out for patients donating plaques 31 and 35.
Figure 1: Sectioning of two separate carotid plaque specimens. Plaque 27 (A) was removed from the right carotid artery of an 86-year-old male with 79% stenosis. Plaque 33 (B) was excised from a 75-year-old female with 50%–59% stenosis. Plaques were removed from −80°C storage and were cut using a scalpel while still frozen into the sections shown in the figures. The segments were photographed before being homogenised and biochemically analysed, the data of which are shown in Figure 2 for plaques 27 and 33.

extremely variable both between plaques and between individual sections of individual plaques, making the overall classification of each plaque difficult. The plaques were crudely classified based on their most significant feature: calcified, thrombotic or thickened intima (Table 1). The thickened intima plaques contained regions of significant lipid deposition. However, many plaques displayed a combination of these characteristics over their length.

Two representative carotid plaques (plaques 27 and 33) are shown in Figure 1. Both plaques had low levels of calcification with distinct regions of what appears to be lipid deposition. The profile of the biochemistry of neopterin, cholesterol, TBARS and α-tocopherol for plaque 27 (Figure 2A–D) appears distinctly different from that seen with plaque 33 (Figure 2E,F). Both these plaques were well-formed carotid branch plaques of similar weight, although plaque 27 had approximately 10-fold higher
Figure 2: Changes in concentration of neopterin, cholesterol, TBARS and tocopherol across two selected carotid plaque specimens. The sections of plaques 27 and 33 shown in Figure 1 were homogenised, and the concentrations of total neopterin (clear bars) and neopterin (black bars) (A and E), cholesterol (B and F), TBARS (C and G), and α-tocopherol (D and H) were measured. Data for plaque 27 are shown in panels A–D and those for plaque 33 in panels E and F.
levels of the analytes measured except for α-tocopherol (Figure 2D). Plaque 33 had very little variation in neopterin or total neopterin (Figure 2E), while plaque 27 had extremely high levels in four distinct sections [3–6], which were separated by regions of low levels (Figure 2A). The highest total neopterin level measured in the study was 18 nmol/g of plaque tissue and was found in a small bifurcation region labelled Section 3 of plaque 27 (Figures 1A3 and 2A). Sections 4, 5 and 6 from this one plaque also had very high levels of total neopterin, measured at between 4 and 7.8 nmol/g (Figure 2A).

Comparison of neopterin levels and locations over all the plaques showed large variations for both total neopterin (Figure 3A) and neopterin (Figure 3B–D). For clarity, the levels of neopterin are shown according to whether the neopterin was decreasing (Figure 3B), not varying (Figure 3C) or fluctuating (Figure 3D) along the length of the plaque. In plaques 21, 26 and 30 only, 7,8-dihydroneopterin was detected as total neopterin. This was most notable in plaque 30 where up to 1 nmol/g of total neopterin was measured with no neopterin, suggesting no oxidation was occurring in this segment at the time of surgery. No neopterin or total neopterin was detected in plaque 19. The extent of the neopterin and total neopterin concentration variation is further demonstrated in Figure 4.

![Figure 3: Neopterin and 7,8-dihydroneopterin concentrations within individual plaque sections. The levels of total neopterin (A) and neopterin (B–D) showed large variation in concentration, so they are displayed on a log scale. For clarity, the levels of neopterin are shown according to whether the neopterin was decreasing (B), not varying (C) or fluctuating (D) along the length of the plaque. No neopterin was detected in plaques 19, 21, 26 and 30, so none is shown.](image-url)
Using multiple correlation analyses, we observed intra- and inter-plaque variation in all analytes. Further inspection of the entire data set failed to show any significant correlation. However, some strong correlations were observed within many of the plaques, but there was no consistency to the pattern observed. A positive significant (p<0.05) correlation between \( \alpha \)-tocopherol and cholesterol was measured for 13 of the 28 plaques, although one plaque gave a significant negative correlation (plaque 27).
The possibility of a trend in the rise and fall in the concentration of the measured biomolecules along the length of the plaque was investigated by converting the analyte concentrations to a log of concentrations and comparing the mean and variance between pre-bifurcation, bifurcation and post-bifurcation of the carotid plaques. This analysis failed to show any significant trend for any of the analytes measured (data not shown). As it was not possible to determine the proximal from the distal ends of the femoral plaque samples after surgery, femoral plaques were excluded from this analysis.

A possible explanation for this range of data is that the plaque tissue is a very dynamic inflammatory site that changes continuously over time. Surgical specimens are therefore a “snapshot” of one particular time. The dynamic nature of the plaques was further explored by culturing live sectioned plaques in tissue culture media within an hour after removal in surgery. Measurement of lactate levels over the first 48 h showed that the plaque tissue segments continued to metabolise the nutrients in the media (data not shown). The addition of γ-interferon caused a large rise in both neopterin and total neopterin for all segments by 48 h (Figure 5, dark bars). The increase in neopterin varied from 0 to 2.5 nmol/(g 24 h) and from 0.7 to 3.7 nmol/(g 24 h) for total neopterin. The total neopterin concentration after 48 h of γ-interferon stimulation ranged from 1 to 4 nmol/(g 24 h). The highest 7,8-dihydroneopterin concentration was calculated as 1.7 nmol/(g 24 h) (Figure 5D and E, Section 1). The rise in neopterin and 7,8-dihydroneopterin concentration in the media shows that these pterins easily diffuse out of the cells and tissues into the media. The effect of γ-interferon on the 7,8-dihydroneopterin generation and oxidation by two plaques was strikingly different. In the cultured plaque (Figure 5A–C), all the 7,8-dihydroneopterin was released as neopterin into the media, so the neopterin percentage against total neopterin was 100% for all segments (Figure 5C, white bars). After 24 h with γ-interferon, 70% of the total neopterin was 7,8-dihydroneopterin in segments 1–3, while it was 100% in segments 4–6. The neopterin levels rose to around 60% of total for all the segments by 48 h after γ-interferon. In contrast, in the second cultured plaque (Figure 5D and E), the neopterin levels remained around 40% of total neopterin throughout the incubation, even after γ-interferon addition (Figure 5F).

Discussion

The amount of neopterin and 7,8-dihydroneopterin measured within the plaque samples and that generated by interferon-stimulated live plaque sections indicate that the likely source of the elevated levels of plasma neopterin in patients with cardiovascular disease (CVD) is macrophages within the atherosclerotic plaques. This does not exclude other sources of neopterin in CVD, as neopterin levels appear to rise even further during acute myocardial infarctions, possibly due to inflammation of the surrounding tissues in response to necrosis [31–33].

Neopterin plasma concentrations range between 10 and 100 pmol/mL, so they are much lower than the range (nmol/g) we observed in the plaques [33, 34]. The concentrations of the pterins in the plaque were similar to the ranges we previously reported in human pus [24]. The ability of 7,8-dihydroneopterin and neopterin to diffuse out of cultured plaques clearly demonstrates that these pterins, when released from the cells, are capable of diffusion through the tissues into the blood.

In 12 of the analysed plaques, the 7,8-dihydroneopterin concentration exceeded 2 μmol/L. We have previously shown that 2 μmol/L of 7,8-dihydroneopterin inhibits LDL oxidation [13], although 50–100 μmol/L is required to prevent oxLDL- or HOCl-induced cell death [16–18]. It is possible that macrophage cell death occurs at a very localised cellular level, so concentrations of 7,8-dihydroneopterin around the cells may reach protective levels.

Stimulation of plaque with γ-interferon clearly showed that some plaques are capable of generating 7,8-dihydroneopterin at antioxidant concentrations (Figure 5), although this stimulation also caused oxidation of 50%–60% of the 7,8-dihydroneopterin to neopterin. This suggests that 7,8-dihydroneopterin may be scavenging significant levels of reactive oxygen species generated within the cultured plaques in response to γ-interferon activation. The large differences seen between the two cultured plaques further show that the plaque is a relatively dynamic environment where the level of 7,8-dihydroneopterin/neopterin may increase or decrease depending on the inflammatory state of any one particular region. The plaque’s inflammation state at the time of surgery therefore greatly affects the measured levels.

In starting this study, we had expected to see clearly defined correlations and trends within the plaques studied. We specifically expected to see a relationship between TBARS, protein-bound DOPA and neopterin. The data instead show that advanced carotid and femoral plaques have highly variable biochemistry and morphology. We failed to find any consistent correlation between the various analytes nor any significant trend in the rise and fall in the concentration of the analytes over the length of individual plaque. Plaques are often described in the literature as being of a particular type [35], yet we
Figure 5: Cultured sections of live atherosclerotic plaque generate neopterin and 7,8-dihydroneopterin after γ-interferon stimulation. Two separate plaques were sectioned into 3-mm-thick rings within an hour of surgical removal and cultured for 4 days in RPMI1640 supplemented with 10% human serum. The data shown in A–C were from sections generated from a plaque removed from a 63-year-old female patient. The plaque was thin walled with heavy calcification in Section 2, which also had regions of ulceration with clot material embedded in the wall. Section 3 included a small bifurcation from the main longitudinal stem of the plaque. The data shown in D–F were from an 82-year-old female patient. Section 2 covers the region of bifurcation, while Section 5 was calcified with some ulceration and haemolysis. Sections 6 also contained a region of ulceration. The cell culture media where the sections were in was removed every 24 h and analysed for neopterin (A and D) and total neopterin (B and E). Following the second media change after 48 h of incubation (white bars), 250 U/mL (A–C) or 500 U/mL (D–F) of γ-interferon was added to stimulate 7,8-dihydroneopterin synthesis and release. The percentage of neopterin to total neopterin was calculated from the data shown. The percentage of neopterin to total neopterin was calculated from the data shown. The legend shown in (A) also applies to (B) and (C), and the legend shown in (D) also applies to (E) and (F).
have observed that the morphology and biochemistry change dramatically over very short distances within individual plaques. This variability and the highly dynamic nature of the inflammatory process appear to be masking the original processes driving plaque formation as measured by the analytes.

Our data do agree with the range of concentrations of the various analytes reported in the literature using whole plaque homogenates. Mean cholesterol levels around 40 μmol/g [36] and ranges of 4–60 μmol/g [37] of tissue have been reported from whole plaque homogenates. Concentrations of TBARS have ranged from 20 to 50 nmol/mg [37, 38], while we observed sections with concentrations from 2 to 130 nmol/mg (Figure 3B). This large within-plaque variability has been previously reported and is similar to the range within the individual plaques in this study. α-Tocopherol has been measured at a mean concentration of 86 nmol/g with a standard deviation of 62 nmol/g in five plaques, which is a large range similar to that reported here [36]. Protein-bound DOPA has been found at 5 and 14 nmol/g of plaque tissue, which is within the range we have observed [39].

This study clearly shows that atherosclerotic plaques are capable of generating significant amounts of 7,8-dihydroneopterin and neopterin that could freely diffuse into the patient’s blood. The levels of the pterins found within plaque tissue and that which can be released from γ-interferon-stimulated plaques are extremely variable. This demonstrates the highly dynamic nature of both 7,8-dihydroneopterin generation and its oxidation to neopterin.

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