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## Immunoglobulins against Tyrosine Nitrated Epitopes in Coronary Artery Disease

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### Abstract

**Background**—Several lines of evidence support a pathophysiological role of immunity in atherosclerosis. Tyrosine nitrated proteins, a footprint of oxygen and nitrogen derived oxidants generated by cells of the immune system, are enriched in atheromatous lesions and in circulation of coronary artery disease (CAD) subjects. However, the consequences of possible immune reactions triggered by the presence of nitrated proteins in subjects with clinically documented atherosclerosis have not been explored.

**Methods and Results**—Specific immunoglobulins that recognize 3-nitrotyrosine epitopes were identified in human lesions, as well as in circulation of CAD subjects. The levels of circulating immunoglobulins against 3-nitrotyrosine epitopes were quantified in CAD patients (n=374) and subjects without CAD (non CAD controls, n=313). A ten-fold increase in the mean level of circulating immunoglobulins against protein-bound 3-nitrotyrosine was documented in the CAD subjects ( $3.75 \pm 1.8 \mu\text{g antibody Eq/mL plasma}$  vs.  $0.36 \pm 0.8 \mu\text{g antibody Eq/mL plasma}$ ), and was strongly associated with angiographic evidence of significant CAD.

**Conclusions**—The results of this cross sectional study suggest that post-translational modification of proteins via nitration within atherosclerotic plaque-laden arteries and in circulation serve as neoepitopes for elaboration of immunoglobulins, thereby providing an association between oxidant production and the activation of the immune system in CAD.

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## Keywords

atherosclerosis; immune system; antibodies; antigens; nitric oxide; free radicals

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## INTRODUCTION

Chronic inflammation, oxidative processes and the activation of the immune system are implicated in the pathogenesis of atherosclerosis.<sup>1-3</sup> The detection and quantification of tyrosine nitrated proteins in human and animal models of atherosclerosis has provided one of the mechanistic links between chronic inflammation and oxidative processes in coronary artery disease (CAD). Tyrosine nitration is a covalent post-translational modification of proteins that arises from the reaction of protein tyrosine residues with nitric oxide-derived oxidants.<sup>4-6</sup> Using mass-spectrometry-based proteomic approaches, site-specific nitration of proteins with pathobiological relevance to CAD have been revealed<sup>7, 8</sup>, while quantification of 3-nitrotyrosine in plasma using stable isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) demonstrated its association with CAD.<sup>9</sup> Despite the potential utility of 3-nitrotyrosine as a biomarker, at the individual protein level, it remains unclear whether protein nitration is responsible for alterations in cellular function that imparts an increased risk for disease development or unfavorable outcomes. Emerging data implicate tyrosine nitration as an activator of immune responses, including the elaboration of immunoglobulins, in inflammatory diseases such as acute lung injury, osteoarthritis, rheumatoid arthritis, and systemic lupus erythematosus.<sup>10-12</sup> Moreover, a possible role of immune modulation by protein nitration can be evoked in other prevalent vascular diseases such as angiotensin II-induced hypertension in which oxidants, T cells and infiltrating monocytes contribute to the disease pathogenesis.<sup>13, 14</sup> In this study, we provide clinical data demonstrating that circulating immunoglobulins that recognize protein-bound 3-nitrotyrosine are increased in CAD subjects and are associated with enhanced risk of angiographic evidence of significant CAD.

## MATERIALS AND METHODS

### Subjects

Stable subjects undergoing elective diagnostic cardiac evaluation with coronary angiography were prospectively recruited to participate. All blood draws were from fasting subjects, venous sampling into EDTA (lavender top) vacutainers prior to heparin administration from sequential consenting subjects at the time of cardiac catheterization. Blood was immediately placed either on ice or within a refrigerator, and subsequently centrifuged for 15 minutes at 10,000 x g at 4°C. Plasma was maintained between 0-4°C and aliquots were frozen at -80 °C within 2 hours of blood draw. Subjects who experienced acute coronary syndrome within 30 days of sample collection or had significant end-organ dysfunction were excluded. All subjects included in the present studies were confirmed to be troponin I (Abbott Architect assay) negative. Angiographic evidence of significant CAD was defined by the presence of at least 50% stenosis in one or more major coronary vessel as judged by at least two cardiologists blinded to patient identification and history. The demographic data for the subjects are reported in the Table 1. Subjects with no history of CAD, significant angiographic (using 50% stenosis as cutoff) evidence of cardiovascular disease, peripheral artery disease, or other significant end organ dysfunction were considered “non-CAD subjects”. The reasons for cardiac catheterization within the study cohort (subjects can have one or more reasons) included: history of positive or indeterminate stress test (46%), evaluation for possible ischemic causes of symptoms (69%), preoperative evaluation (14%),

and history of cardiomyopathy (3%). All study participants gave informed consent, and the study was approved by the Cleveland Clinic Institutional Review Board.

## Materials

Free 3-nitrotyrosine and the synthetic octapeptide containing two 3-nitrotyrosine residues (CGnitroYGGGnitroYG) (CPC Scientific, San Jose, CA) were conjugated to horseradish peroxidase (HRP) following a previously published one step procedure.<sup>10</sup> Affinity purified polyclonal anti-nitrotyrosine antibodies were generated and characterized extensively<sup>15, 16, 8</sup> and used as reference immunoglobulin. Synthetic peptides comprising amino acids 213-219 of human apolipoprotein A-I sequence (<sup>213</sup>LAEYHAK<sup>219</sup>) with either tyrosine in position 216 or 3-nitrotyrosine (<sup>213</sup>LAEnitroYHAK<sup>219</sup>) were used for ligand competition. Human fibrinogen (America Diagnostica, Stamford, CT), fibronectin and ceruloplasmin were nitrated by addition of 3 consecutive bolus additions of peroxydinitrite (final concentration 100  $\mu$ M) in 0.1 M phosphate buffer containing 100  $\mu$ M DTPA and 25 mM bicarbonate, pH 7.2. These proteins were chemically nitrated and used to screen for the circulating immunoglobulin isotype.

## Immunohistochemistry

To probe for tyrosine nitrated proteins sections from human carotid atheromatous lesions were fixed in cold methanol-acetone solution for 20 min at  $-20^{\circ}\text{C}$ . Staining for tyrosine-nitrated proteins was performed using rabbit polyclonal affinity purified anti-nitrotyrosine antibodies (7  $\mu\text{g}/\text{mL}$ ) raised against the synthetic peptide described previously.<sup>15,16</sup> A goat anti-rabbit secondary antibody conjugated to HRP (Biorad, Hercules, CA) was used for signal detection. As control for the specificity of the antibody binding, the anti-3-nitrotyrosine antibody was pre-incubated with 250  $\mu\text{M}$  3-nitrotyrosine octapeptide or with 250  $\mu\text{M}$  of the corresponding tyrosine octapeptide for 1 hour at room temperature. Other control experiments included; pre-incubation with 2.5 mM free 3-nitrotyrosine, reduction of tissue nitrotyrosine to aminotyrosine using 0.5 M dithionite in 0.1 N sodium hydroxide for 20 min at room temperature, as well as omission of the primary antibody. Oil red-O, trichrome staining and 3,3'-diaminobenzidine (DAB)-chromogenic detection of immunoglobulins was performed in human lesions fixed in 4% paraformaldehyde as described previously.<sup>8, 15,16</sup>

## Affinity purification of immunoglobulins

Proteins that bind to 3-nitrotyrosine were affinity isolated using an Amino Link Plus Immobilization Kit (Pierce, Rockford, IL), as described previously with minor modifications.<sup>8</sup> Briefly, 10 mg of plasma proteins (combining plasma from three CAD patients) were diluted in binding buffer (50 mM potassium phosphate, 400 mM NaCl, pH 7.5), applied to the column, and incubated for 1 hour, at room temperature. Unbound protein fractions were eluted by centrifugation with binding buffer. Bound proteins were eluted with 0.1 M citrate, pH 2.5, and neutralized with 1 M Tris, pH 9. Bound fractions were concentrated and buffer exchanged using Millipore 10 kDa centrifugal filter units (Millipore Corp., Billerica, MA). The protein fractions were separated on 10% SDS-PAGE and transferred to Immobilon-P PVDF membranes (Millipore Corp., Bedford, MA), at 100V and  $4^{\circ}\text{C}$ . After blocking with 3% BSA in TBS-T, the membranes were incubated with either goat anti-human IgG-IRDye 800 CW (Rockland Immunochemicals, Inc. Boyertown, PA) or mouse anti-human IgM (1:2500, Sigma, St. Louis, MO) plus goat anti-mouse IgG conjugated to Alexa Fluor 680 (1:5000, Invitrogen, Eugene, OR) in blocking buffer. Images were obtained with an Odyssey near infrared imaging system (LI-COR, Inc. Lincoln, NE) at 700 and 800 nm.

Human arterial tissue was obtained from the National Disease Resource Institute (Philadelphia, PA) or the Cleveland Clinic. Tissues (approximately 1 gram) were cleaned of connective tissue and blood, snap frozen in liquid nitrogen, and pulverized in the frozen state and stored at  $-80^{\circ}\text{C}$  until use. Frozen tissue powder was thawed in 5 ml extraction buffer containing protease inhibitor cocktail (Sigma, St Louis MO), and further macerated with a Tissue-Tek homogenizer operated on full power for 10 seconds. This procedure was repeated 4 times per tissue sample. Tissue extracts were centrifuged at  $700 \times g$  for 5 min and  $21,000 \times g$  twice for 10 min to remove particulate material. Supernatants were combined and loaded to appropriate columns for affinity enrichment.

### Isotype determination

ELISA methodologies were developed to determine the isotype and the sub-isotype of the circulating immunoglobulin that recognize protein-bound 3-nitrotyrosine. First, to differentiate between IgG and IgM,  $100 \mu\text{l}$  of  $10 \mu\text{g/ml}$  of the synthetic 3-nitrotyrosine octapeptide described above was diluted in 50 mM bicarbonate buffer, pH 9 and added in the wells of 96 well plates (MAXI-SORP, Nunc, Roskilde, Denmark). After overnight incubation at  $4^{\circ}\text{C}$  the plates were washed and incubated in the presence of  $100 \mu\text{l/well}$  3% BSA in TBS-T (working buffer), for 2 hr at room temperature. Human plasma from CAD patients (5 mg of plasma protein/mL) were added to each well and incubated for 2 hr at room temperature. Anti-human IgG and anti-human IgM HRP-conjugated antibodies (Sigma, Saint Louis, MO) diluted to 1:5,000 in working buffer were added to appropriate series of wells. Peroxidase activity bound to each well was determined with 3,3',5,5'-tetramethylbenzidine (TMB, Pierce Biotechnology, Inc., Rockford, IL) substrate.

To determine the IgG subclass of the antibody, microplates were coated with  $100 \mu\text{L}$  of  $5 \mu\text{g/mL}$  solution of nitrated and native ceruloplasmin, fibronectin, and fibrinogen. The plates were incubated overnight at  $4^{\circ}\text{C}$ . After blocking,  $100 \mu\text{l}$  of human plasma (diluted 1:40) alone or in the presence of  $10 \mu\text{M}$  synthetic 3-nitrotyrosine octapeptide (specificity control) in blocking buffer were added to each well and incubated for 1hr, followed by the addition of  $100 \mu\text{l/well}$  of 1:2,000 dilutions of monoclonal HRP-conjugated anti-human IgG 1, 2, 3, and 4 (Sigma, Saint Louis, MO). Absorbance was measured at 450 nm on a micro-plate reader (Spectra-Max 250, Molecular Devices Corp., Sunnyvale, CA). The background absorbance obtained with unmodified proteins was subtracted from the absorbance of the samples that reacted with the nitrated protein antigens.

### Ligand competition ELISA

A competition ELISA described previously was implemented to determine the levels and specificity of circulating immunoglobulins that recognize 3-nitrotyrosine in human plasma.<sup>10</sup> Briefly, each well of a 96-well microtiter plate (MAXI-SORP, Nunc, Roskilde, Denmark) was coated with  $50 \mu\text{l}$  of serial dilutions of human plasma (0.02-10 mg of protein/ml) or a single dilution (as indicated in the appropriate figure legend), in 50 mM sodium bicarbonate buffer, pH 9. After blocking, 75 nM horseradish peroxidase (HRP)-conjugated 3-nitrotyrosine or a HRP-conjugated synthetic octapeptide containing two 3-nitrotyrosine residues (CGnitroYGGGnitroYG) were added in 0.1% BSA in TRIS buffered saline with 0.05% tween-20 (TBS-T), and incubated for 1 hr. Peroxidase activity bound to each well was determined using the  $\text{H}_2\text{O}_2$ -peroxidase mediated oxidation of TMB measuring absorbance at 450 nm. To test the specificity of detection the absorbance at 450 nm was competed in each sample by inclusion of excess free, unlabeled, 3-nitrotyrosine (2.5 mM). In some experiments the CGnitroYGGGnitroY-G octapeptide was used as competitor.

A polyclonal rabbit anti-nitrotyrosine IgG antibody (0.08-40  $\mu\text{g}$  of protein/ml) characterized in detail previously<sup>15, 16</sup> was run each day along with plasma samples. The antibody titer in

each sample was calculated using the absorbance at 450 nm of the samples (Abs<sub>450nm</sub>), and the average of the parameters of the dose-response curve obtained with the polyclonal anti-nitrotyrosine antibody using the following equation:

$$\text{Antibody titer} = 200C \left( \frac{A - D}{\text{Abs}_{450\text{nm}} - D} - 1 \right)^{1/B};$$

where 200 represents the sample dilution and the average values for the parameters A, B, C, and D were 0.07, 2.21, 0.04, and 1.57, respectively, denoting minimal absorbance, slope at the linear part of the curve, the dose at the central point, and the maximal absorbance, respectively. Antibody titers in human plasma are therefore expressed as  $\mu\text{g}$  equivalents of the polyclonal antibody per mL of plasma ( $\mu\text{g}$  Ab Eq/mL plasma).

### Isolation of nitrated proteins for mass spectrometry

Proteins bearing the 3-nitrotyrosine epitope were affinity captured from human CAD plasma by using the polyclonal anti-3-nitrotyrosine antibody conjugated to a recombinant protein A column (Pierce, Rockford, IL). Pooled plasma from six CAD patients (14 mg total protein) were loaded into the column in 0.1 M Hepes, pH 7.4 (binding buffer), and incubated overnight at 4° C. The column was washed successively with 10 mL of the following solutions: binding buffer, 0.5 M NaCl, and binding buffer again. Bound fractions were eluted with 10 mL of 0.1 M glycine, pH 2.5, containing 0.15 M NaCl. After concentration and buffer exchange using Centriprep YM-10 filters (Millipore Corp., Billerica, MA), proteins were separated on 10% SDS-PAGE, and stained with colloidal blue (Invitrogen, Carlsbad, CA). An identical gel was transferred to PVDF membrane for immunodetection of protein 3-nitrotyrosine using the polyclonal anti-nitrotyrosine antibody.

### Gel-LC-MS/MS analysis

Colloidal blue stained gels were cut into 11 × 2mm slices and in-gel digested with trypsin as previously described.<sup>8</sup> Tryptic peptide digests were analyzed by a LTQ linear IT mass spectrometer (Thermo Electron, San Jose, CA) coupled to an Eksigent 2D LC system (Eksigent Technologies, Livermore, CA) and autosampler. Buffers A and B were 0.1 % formic acid/1 % methanol and 80% acetonitrile/0.1% formic acid/1 % methanol, respectively. Peptides were loaded isocratically onto a C18 trap column (75  $\mu\text{m}$  i.d. x 25 mm; New Objective Proteopep 2) at a flow rate of 1  $\mu\text{L}$  per minute in 2% B. Peptides were then eluted onto a C18 analytical column (75  $\mu\text{m}$  i.d. x 150 mm; New Objective Proteopep 2). A linear gradient was then initiated at a flow rate of 300 nL per minute for 90 min from 3-40% B. The mass spectrometer was set to repetitively scan  $m/z$  from 375 to 1600 followed by data-dependent MS/MS scans on the five most abundant ions with dynamic exclusion enabled.

### Generation and evaluation of SEQUEST peptide assignments

DTA files were generated from the MS/MS spectra extracted from RAW data files (intensity threshold of 1000; minimum ion count of 50) and processed by the ZSA and Correction algorithms of the SEQUEST Browser program. DTA files were submitted to Sorcerer-SEQUEST (ver. 3.11, rev 11; Sagen Research, San Jose, CA) using the following parameters: Database searching was performed against a Uniprot database containing *Homo sapiens* sequences from Swiss-Prot plus common contaminants, which were then reversed and appended to the forward sequences (91,522 entries). The database was indexed with the following parameters: mass range of 600 - 3500, tryptic cleavages with a maximum of 1 missed cleavage and static modifications of cysteine by carboxyamidomethylation (+57

amu). The DTA files were searched with a 2.0 amu peptide mass tolerance, 1.0 amu fragment ion mass tolerance, and variable modification of methionine (+16 amu). Potential sequence-to-spectrum peptide assignments generated by Sorcerer-SEQUEST were loaded into Scaffold (version 2.2; Proteome Software, Portland, OR) to validate protein identifications and perform manual inspection of MS/MS spectra containing 3-nitrotyrosine. Protein identifications were accepted at a threshold of 99 % protein confidence with 2 unique peptides at 80 % confidence. From these proteins, manual inspection of 3-nitrotyrosine-containing MS/MS spectra were performed using the following criteria: (1) assignment of the majority of fragment ion abundance, (2) 3-nitrotyrosine (+45 amu) modification supported by either y- or b- ions series ( 5 consecutive fragments), and (3) correctly assigned charge state and diagnostic markers, such as N-terminal proline, C-terminus aliphatic amino acids, and loss of H<sub>2</sub>O/ammonia consistent with amino acid sequence.

### Statistical Analysis

The Student's t-test or Wilcoxon-Rank sum test for continuous variables and chi-square test for categorical variables were used to examine the difference between the groups (CAD vs. non-CAD). The relationship between plasma immunoglobulin levels and risk for having CAD was determined by calculating Odds ratio (OR) and 95% confidence intervals (95%CI) using multiple logistic regression treating immunoglobulin levels against protein-bound nitrotyrosine as a continuous variable. Models adjusted for traditional risk factors (age, gender, systolic blood pressure, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, smoking), or traditional risk factors plus history of myocardial infarction (MI), and medications (aspirin, ACE, statin, beta-blockers). Cubic spline curves with 95% confidence intervals were constructed to illustrate the relationship between anti-nitrotyrosine immunoglobulin levels and odds ratio of angiographic evidence of significant CAD. All analyses were performed using R 2.13.1 (Vienna, Austria) and p-values <0.05 were considered statistically significant.

## RESULTS

### Nitrated proteins in human atherosclerotic lesions and plasma

The presence of nitrated proteins in atherosclerotic lesions and/or plasma of CAD patients could serve as neopeptides, triggering an immune response and the elaboration of immunoglobulins. Therefore, we first sought to document the presence of nitrated proteins by immunohistochemistry in human lesions and by mass spectrometry in lesion extracts as well as plasma of subjects with CAD. A typical atherosclerotic lesion in human carotid artery stained with Oil red O is depicted in Figures 1 A and B. The immunofluorescence images after staining for nitrated protein epitopes is depicted in Figures 1C and D indicated the presence of nitrated proteins within the lesion, consistent with previous studies in human atherosclerotic lesions.<sup>6</sup> The specificity of the anti-nitrotyrosine antibodies used for staining was confirmed by competition experiments with excess of the nitrated octapeptide (CGnitroYGGGnitroYG). Moreover, staining with anti-human IgG also revealed the presence of immunoglobulins in the lesions (Fig. 1G). Nitrated proteins extracted from atherosclerotic lesions and plasma were affinity enriched using the previously characterized polyclonal anti-nitrotyrosine antibody.<sup>15, 16</sup> As a control, lesion extracts were incubated with a non-specific rabbit immunoglobulin under the same conditions (representative western blots confirming selective enrichment are depicted in supplementary figure 1). The bound fractions were separated by 1D SDS-PAGE, and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Only proteins that were identified in the bound fraction of anti-nitrotyrosine-enriched samples are reported (Supplementary Table 1). Within the lesion, several proteins that have been previously reported to be modified by

tyrosine nitration such as apolipoprotein AI (apoA-I), beta chain of fibrinogen,  $\alpha$ 1-antitrypsin and complement were identified.<sup>7, 17-19</sup> A similar analysis in human plasma revealed for the first time the presence of specific peptide sequences that contained the nitrated tyrosine residue. Several of these sequences belong to immunoglobulins (supplementary table 2). Interestingly one specific tyrosine nitrated peptide [(K)SGTASVVCLLNNFnitroYPR(E)] from human immunoglobulin was also identified in the plasma of atherosclerosis rodent models; specifically, the low-density lipoprotein receptor (LDLr) and apobec-1 null double-knockout mice, as well as the LDLr/apobec-1 knockout mice lacking apoA-I (LA-apoA-I<sup>-/-</sup>).<sup>8</sup> Collectively the data indicate that nitrated proteins are present in human atherosclerotic lesions as well as plasma of subjects with CAD and could serve as antigenic neopeptides to trigger the activation of the immune system.

### **Affinity chromatography identified immunoglobulins that recognize 3-nitrotyrosine and nitrated proteins**

Protein extracts of human aorta atherosclerotic lesions were allowed to bind to affinity columns conjugated with 3-nitrotyrosine. After binding and exhaustive washing, the bound fractions were eluted, separated by SDS-PAGE and analyzed by Western blot using anti-human IgG antibodies (Fig. 2A). The presence of immunoglobulins that bound to 3-nitrotyrosine was documented in the lesion tissue extract. As controls, tissue from the same aorta free of lesion was used and analyzed in parallel. Equivalent loading of extracts derived from aorta segments from the same tissue, but without visible atherosclerotic lesions, did not show immunoreactive bands for IgG in the bound fraction eluted from the 3-nitrotyrosine affinity column (Fig. 2A).

Pooled plasma from CAD subjects was allowed to bind to affinity columns that were made by conjugating either the nitrated tyrosine octapeptide (CGnitroYGGGnitroYG) or the equivalent tyrosine peptide (CGYGGGYG). The data in Figure 2B indicate the presence of IgG in the human plasma of CAD subjects that specifically bind the nitrated tyrosine peptide but not the tyrosine peptide. Plasma proteins recovered in the bound fraction from the nitrated tyrosine octapeptide affinity column also showed immunoreactivity toward human IgM antibodies. By employing an ELISA in which the 3-nitrotyrosine octapeptide was used to bind the immunoglobulins and the respective anti-human immunoglobulin to develop, we quantified the relative levels of IgG and IgM in human CAD subjects plasma. Plasma immunoglobulins that recognize the nitrotyrosine modification were predominantly IgG, since the signal was nearly 5-fold higher for IgG as compared to IgM ( $1.4 \pm 0.3$  and  $0.3 \pm 0.3$  average absorbance respectively, Fig. 3a). Therefore, subsequent studies focused on quantifying the levels of circulating IgG immunoglobulin isoform in subjects. The IgG present in human plasma of CAD subjects was further characterized by isotyping using an ELISA in which potential endogenous 3-nitrotyrosine containing proteins: nitrated ceruloplasmin, nitrated fibronectin, and nitrated fibrinogen were used as antigens. The predominant isotype that recognized preferentially the nitrated proteins was IgG2 (Fig 3B).

### **ELISA development to quantify plasma levels of anti-nitrotyrosine immunoglobulins in non-CAD and CAD subjects**

We next sought to determine whether plasma immunoglobulins that recognize the neopeptide 3-nitrotyrosine are more prevalent within subjects with CAD. For these studies, we modified a competition ELISA described previously for use in human plasma and mouse models of atherosclerosis.<sup>10, 8</sup> Typical antigen-antibody binding curves using the nitrotyrosine-containing octapeptide conjugated to HRP are shown in Figure 3, panel C. No binding was observed using the same tyrosine-containing octapeptide. The specificity of binding was confirmed by competing the binding of the 3-nitrotyrosine peptide-conjugated HRP with excess free 3-nitrotyrosine (2.5 mM), as well as un-conjugated nitrotyrosine

peptide (75  $\mu$ M). In all subsequent analyses the difference between the absorbance in the absence versus presence of excess 3-nitrotyrosine (2.5 mM), considered as background, was used to calculate the titer of circulating anti-3-nitrotyrosine immunoglobulins. Furthermore, the binding of immunoglobulins to the HRP-conjugated nitrotyrosine peptide was competed by a peptide derived from apoA-I ( $^{213}$ LAEnitroYHAK $^{219}$ ) containing 3-nitrotyrosine in place of tyrosine in position 216. Maximal inhibition was obtained at 1  $\mu$ M  $^{213}$ LAEnitroYHAK $^{219}$  (Fig. 3, panel D). At the same concentration, the unmodified apoA-I peptide,  $^{213}$ LAEYHAK $^{219}$ , was unable to compete (Fig. 3, panel D) confirming the specificity of the immunoglobulins towards protein tyrosine nitrated epitopes.

### Plasma levels of antibodies that recognize protein nitrotyrosine are increased in subjects with CAD

Plasma levels of anti-3-nitrotyrosine immunoglobulins were quantified in subjects with and without CAD as documented during elective cardiac evaluations, as described under Methods. Subjects with clinically documented CAD had greater than ten times higher levels of anti-3-nitrotyrosine immunoglobulins ( $3.75 \pm 1.8 \mu\text{g Ab Eq/mL plasma}$ ) as compared to non-CAD individuals ( $0.36 \pm 0.8 \mu\text{g Eq/mL plasma}$ ;  $p < 0.001$  for comparison with CAD). Interestingly, subjects with angiographic evidence of significantly-obstructed (> 50% stenosis) CAD showed increased plasma anti-3-nitrotyrosine immunoglobulin titers compared to non-CAD subjects, regardless of the number of coronary arteries affected by CAD (Fig. 4, panel B). A striking dose-dependent positive association was observed between plasma levels of anti-nitrotyrosine IgG and angiographic evidence of significant CAD; in fact, because the non-CAD cohort had very low levels of anti-3-nitrotyrosine immunoglobulins (non-detectable in 76 % of the samples), a remarkably large odds ratio was observed for anti-3-nitrotyrosine IgG titer versus the presence of significant angiographic evidence of CAD (Fig. 4, panel C). Multilogistic regression analyses indicated that the robust association of immunoglobulin levels against protein 3-nitrotyrosine with cardiovascular risk remains significant even following adjustments for multiple traditional risk factors, including age, gender, systolic blood pressure, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, smoking, history of MI and medications (aspirin, angiotensin converting enzyme inhibitors, statin, beta-blockers) (Table 2).

## DISCUSSION

Clinical evidence and data from animal models have identified various forms of oxidized lipoproteins and heat shock proteins as immunogens that induce adaptive immune responses, including antibody elaboration, that modulate atherosclerotic lesion formation in experimental models of atherosclerosis.<sup>20-29</sup> Most studies have predominantly focused on covalent adducts formed by addition of electrophilic lipid peroxidation products to proteins and their relationship to the development and progression of atherosclerosis.<sup>20-26</sup> Moreover, the levels of circulating IgM that recognized oxidized forms of low density lipoprotein with covalently adducted lipid oxidation products have been reported to correlate with severity of atherosclerosis in mice models and in some studies with the progression of atherosclerosis in coronary or carotid vascular beds, or with risk for myocardial infarction and death in humans.<sup>25</sup> Collectively these data highlight the critical importance of understanding the immune system responses in CAD and raise the possibility of novel therapeutic treatments for atherosclerosis.<sup>30, 31</sup> The data also underscores the need for identifying antigens and immune responses that are mechanistically linked to the underlying pathophysiology of cardiovascular diseases.

Protein tyrosine nitration is considered to be a post-translational modification reflective of inflammatory-mediated oxidative processes involving nitric oxide-derived oxidants. Although the ability of nitrated proteins to induce the generation of specific antibodies in



experimental animals has long been known<sup>15, 16, 32-36</sup>, the contribution of this post-translational protein modification as a trigger of immune reactions in pathology has just recently been explored.<sup>10-12</sup> In this study we demonstrated for the first time the association of CAD, the most common and life threatening cardiovascular condition, with increased plasma levels of immunoglobulins reactive against proteins modified by nitration. The binding of plasma immunoglobulins to target 3-nitrotyrosine-containing “bait” (HRP conjugated to 3-nitrotyrosine or 3-nitrotyrosine containing octapeptide) served as a competition-based ELISA to quantify autoantibody titers for 3-nitrotyrosine recognizing immunoglobulins within subject’s plasma. The specificity of the methodology was confirmed by numerous approaches, including demonstration that the 3-nitrotyrosine binding activity in plasma was specifically outcompeted by free 3-nitrotyrosine, as well as by 3-nitrotyrosine-modified peptides and proteins, ruling out nonspecific binding. In particular, a nitrated peptide reflecting an endogenous nitrated tyrosine residue identified in apoA-I recovered from human atherosclerotic plaque<sup>7</sup>, but not the native tyrosine containing peptide, was able to out-compete the binding of the 3-nitrotyrosine-coupled HRP “bait” to immunoglobulins. Moreover, the circulating immunoglobulins were able to recognize nitrated fibrinogen and other nitrated plasma proteins but not the native counterparts (Fig. 3B). These data support a possible specific antigenic role of those endogenous nitrated proteins as the neo-epitope that triggers anti-nitrotyrosine immunoglobulins. It is remarkable that both of these proteins (apoA1 and fibrinogen) have been previously reported to be selectively nitrated in plasma from CAD patients relative to healthy controls, and that nitration of these particular proteins may have a potential functional role in cardiovascular events. In the case of fibrinogen, nitration increases the rate as well as the extent of fibrin cloth formation<sup>17, 18</sup> potentially leading to enhanced thrombotic risk and adverse cardiovascular events. On the other hand, 3-nitrotyrosine modified apoA-I was found in serum and human atherosclerotic lesions. Further, exposure of apoA-1 to myeloperoxidase, an enzymatic source of nitric oxide-derived oxidants, creates a dysfunctional form of HDL with diminished ABCA1-dependent cholesterol efflux capacity and reduced lecithin cholesterol acyl transferase activity.<sup>7, 37</sup> The molecular mechanisms for the production of immunoglobulins with specific and selective epitope recognition for 3-nitrotyrosine have been explored in mouse models.<sup>35, 36</sup> Termination of self-tolerance and escaping of negative selection after active immunization with nitrated peptides was documented.<sup>35, 38</sup> Moreover, monoclonal antibodies that recognize only nitrated  $\alpha$ -synuclein (including stereospecific clones that recognized one of the four nitrated tyrosine residues in  $\alpha$ -synuclein) but not the unmodified protein or other tyrosine-nitrated proteins<sup>32</sup>, as well as antibodies recognizing only nitrated Mn Superoxide dismutase<sup>33</sup>, and only nitrated tau<sup>34</sup> have been reported. In one model of mouse neurodegeneration the specific immune response to nitrated  $\alpha$ -synuclein produced a vigorous immunoinflammatory response that led to the degeneration of dopamine-producing neurons.<sup>39</sup>

The pathophysiological relevance of circulating immunoglobulins targeting protein-3-nitrotyrosine remains to be determined. Indeed, despite the strikingly elevated levels of anti-3-nitrotyrosine antibodies in CAD subjects, it is possible that the antibody response within atherosclerotic plaque may promote a protective role, with immunoglobulins targeting proteins bearing potentially threatening modifications for destruction. Further, the relatively high risk cohort of subjects examined (those undergoing elective diagnostic cardiac evaluations) makes the results of the present study of unclear relevance when translated to a community based screen. It also remains to be determined whether elevated levels of 3-nitrotyrosine targeted antibodies are increased in subjects prior to clinically overt development of atherosclerotic CAD, and whether increased levels may serve as a sentinel of increased risk of major adverse cardiac events or not. Further studies are needed to both identify the role; if any, of 3-nitrotyrosine immunoglobulins in CAD pathophysiology, as

well as whether anti-3-nitrotyrosine immunoglobulin titer carries prognostic value and is modulated by CAD targeting therapies.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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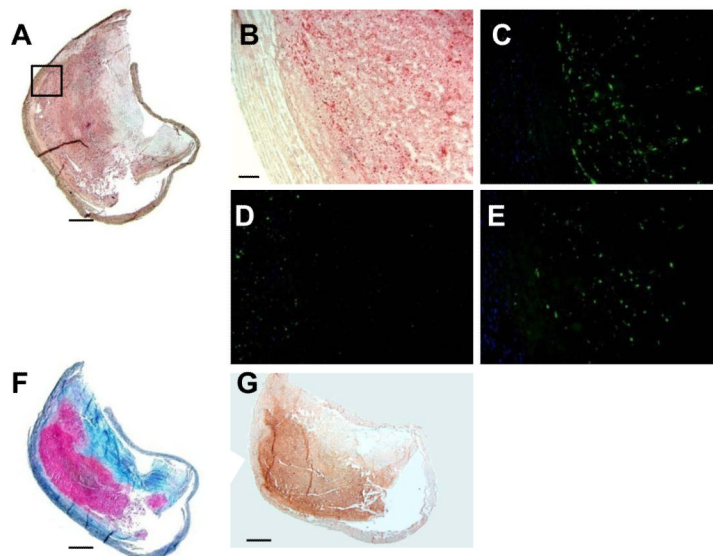
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Clinical evidence and data from animal models have identified a pathophysiological role of immunity in atherosclerosis. In this study we have identified and quantified specific immunoglobulins that recognize nitrated tyrosine epitopes in human atherosclerotic lesions as well as in circulation of subjects with clinically documented coronary artery disease (CAD). Protein tyrosine nitration is a post-translational modification reflective of inflammatory-mediated oxidative processes involving nitric oxide-derived oxidants that is known to elicit the elaboration of immunoglobulins in animal models. A highly significant ten-fold increase in the mean level of circulating immunoglobulins against protein 3-nitrotyrosine was documented in the CAD subjects. The levels of these specific immunoglobulins were positively associated with increased odds ratio for the presence of angiographic evidence of significant CAD. Further studies are needed to explore the pathophysiological relevance of circulating immunoglobulins against nitrated proteins in CAD pathophysiology. Additional studies are also required to test whether anti-3-nitrotyrosine immunoglobulin titer carries prognostic value and is modulated by CAD targeting therapies.

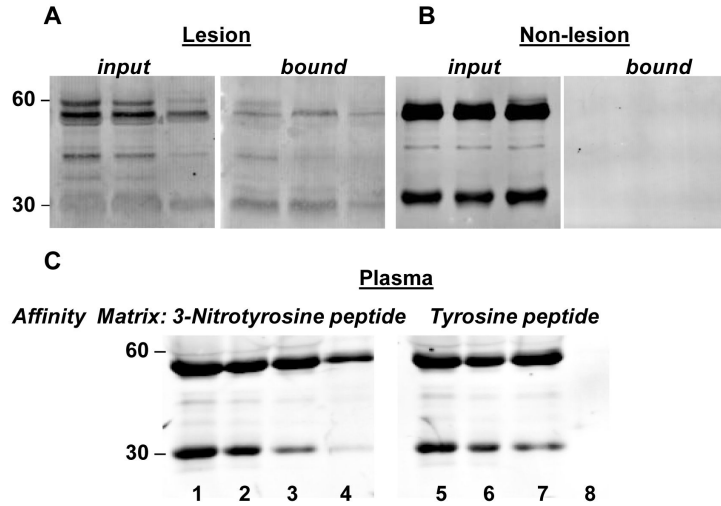
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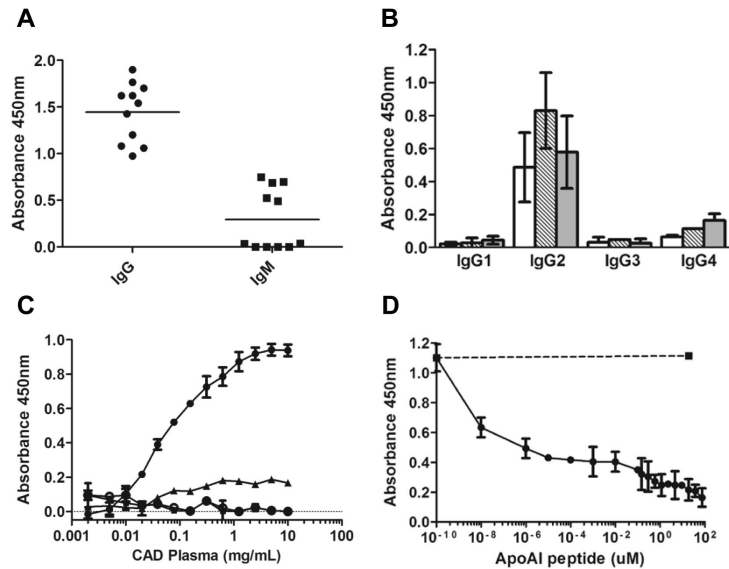
**Figure 1. Immunohistochemical detection of nitrated proteins in human atherosclerotic lesions**  
 Panel A and B: Oil red O staining of the atherosclerotic lesion in human carotid. (A) Low magnification (1.25x) and (B) high magnification (20x). Panels C-E: Serial cross sections were incubated with polyclonal anti-3-nitrotyrosine antibody (C). The immunoreactive nitrated proteins were visualized in the lesions by green fluorescence after incubation with secondary anti-rabbit/Alexa Fluor 488 antibody. Immunoreactivity was reduced by competition of the primary anti-3-nitrotyrosine antibody binding with the 250  $\mu\text{M}$  3-nitrotyrosine-containing octapeptide (D) but not by 250  $\mu\text{M}$  tyrosine-containing octapeptide (E). Nuclei are stained blue with DAPI. The results are representative of staining 2 different human lesions. Panel F and G: Trichrome staining of the carotid lesion (F) and immunostaining with anti-human IgG antibody revealed the presence of IgG immunoglobulins within and around the lesion (G). Immunoreactivity (brown color) was developed with DAB. Bars indicate 100  $\mu\text{m}$ .



**Figure 2. Affinity detection and isotyping of anti-nitrotyrosine immunoglobulins from aortic lesions and CAD plasma**

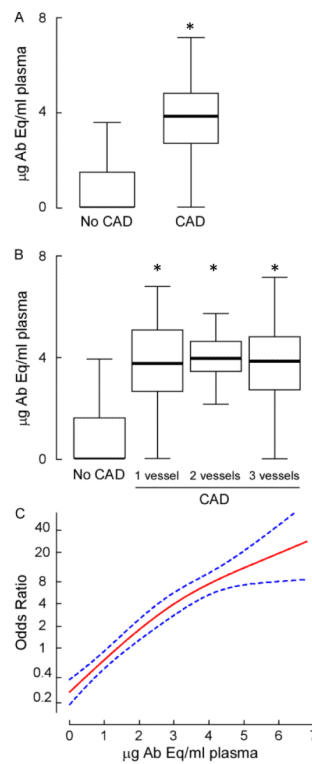
*Panel A:* Representative western blots from three different atherosclerotic lesion extracts (input) and bound fractions eluted from a 3-nitrotyrosine affinity column using anti-human IgG antibodies. *Panel B:* Representative western blots from the same vessels as in Panel A using the equivalent protein extracts from the same tissue without visible lesion.

Immunoreactivity is observed only in the fractions originating from lesion areas. *Panel C:* Affinity captured plasma proteins using either immobilized 3-nitrotyrosine containing octapeptide (Lanes 1-4) or tyrosine containing octapeptide (Lanes 5-8) probed with polyclonal anti-human IgG. Lane 1 and 5, un-fractionated input plasma; lane 2 and 6, unbound fraction; lane 3 and 7, wash fractions; lane 4 and 8, bound fractions eluted with 0.1 M citrate buffer, pH 2.5. Data are representative of six independent experiments with similar results.



**Figure 3. Screening for circulating immunoglobulins that recognize 3-nitrotyrosine**  
*Panel A:* IgG and IgM levels in human plasma were measured by ELISA in ten randomly selected CAD plasma subjects, using the 3-nitrotyrosine octapeptide to bind the immunoglobulins and the respective anti-human immunoglobulin for detection. *Panel B:* IgG subclass was determined by ELISA in three independent CAD plasma samples, using nitrated ceruloplasmin (white bars), nitrated fibronectin (dashed bars) and nitrated fibrinogen (grey bars) as capturing antigen, and HRP-conjugated mouse anti-human IgG 1, IgG2, IgG 3, and IgG 4 for detection. *Panel C:* Typical antigen-antibody binding curves from 3 randomly selected CAD subjects each performed in triplicate, using as ligand the 3-nitrotyrosine-octapeptide conjugated to HRP (●). No binding was observed using the same tyrosine-containing octapeptide conjugated to HRP (○). The binding of the 3-nitrotyrosine-octapeptide conjugated to HRP was eliminated with 75 μM unlabeled 3-nitrotyrosine-octapeptide (▲) or by 250 μM 3-nitrotyrosine (■). *Panel D:* The binding of the 3-nitrotyrosine-octapeptide conjugated to HRP to 3 different plasma samples, each performed in triplicate, was also eliminated with increasing concentrations of apolipoprotein A-I peptide 213-219 (<sup>213</sup>L-A-E-nitroY-H-A-K<sup>219</sup>) which contained 3-nitrotyrosine in position 216 (●) but not by the same peptide that contained tyrosine in position 216 (■). Data reports mean ± standard deviation.





#### Figure 4. Anti-nitrotyrosine circulating immunoglobulins in CAD

*Panel A:* Plasma levels of anti-3-nitrotyrosine circulating immunoglobulins were quantified in clinically documented CAD subjects vs. subjects without significant angiographic evidence of CAD. Antibody equivalents were calculated from standard curves performed with a polyclonal anti-nitrotyrosine antibody. *Panel A and B:* Box-whisker plots of immunoglobulin levels encompass the 25<sup>th</sup> and 75<sup>th</sup> percentiles and lines within boxes represent median values. Bars represent the 2.5<sup>th</sup> and 97.5<sup>th</sup> percentiles. Statistically significant differences in immunoglobulin content were found between CAD and non-CAD ( $p < 0.0001$ , Kruskal-Wallis test). *Panel C:* Odds ratio and 95% confidence interval (dotted lines) for the relationship between plasma levels of anti-nitrotyrosine immunoglobulins and angiographic evidence of significant CAD were calculated as described under Methods.

**Table 1**

## Baseline Subject Characteristics by CAD status

	All subjects (n=687)	Non-CAD (n=313)	With CAD (n=374)	P
<b>Demographics and Cardiovascular Risk Factors</b>				
Age (years)	68±10	61±8	70±9	<0.001
Male (%)	67	37	81	<0.001
Diabetes mellitus (%)	37	17	46	<0.001
Hypertension (%)	73	56	80	<0.001
Smokers (Former/Current, %)	67	52	73	<0.001
Prior myocardial infarction (%)	32	0	48	<0.001
<b>Laboratory Data</b>				
LDL cholesterol (mg/dL)	96(79-119)	110(90-130)	91(76-112)	<0.001
HDL cholesterol (mg/dL)	35(28-41)	39(34-50)	32(27-39)	<0.001
Triglycerides (mg/dL)	115(86-159)	104(79-143)	119(90-169)	0.006
Total leukocyte count (WBCP/hpf)	6.12(5.117.37)	5.87(4.61-6.95)	6.24(5.21-7.57)	0.003
Uric acid (mg/dL)	6.3(5.1-7.3)	5.6(4.7-6.7)	6.6(5.5-7.6)	0.003
hsCRP (mg/L)	2.53(1.076.66)	1.94(0.85-5.29)	2.83(1.24-7.08)	0.004
Myeloperoxidase (pg/mL)	114(74-246)	104(70-167)	124(80-286)	0.001
Creatinine clearance (ml/min/1.73m <sup>2</sup> )	88(66-110)	97(83-122)	79(62-106)	<0.001
<b>Baseline Medications</b>				
Aspirin	74	60	81	<0.001
Beta-adrenergic blockers	58	47	62	0.002
Angiotensin converting enzyme inhibitors	53	34	61	<0.001
Statin therapy	54	26	67	<0.001

Values expressed in mean ± standard deviation or median (interquartile range). Abbreviations: LDL = low-density lipoprotein; HDL = high-density lipoprotein; hsCRP = high-sensitivity C-reactive protein.

**Table 2**

Relationship between immunoglobulin against 3-nitrotyrosine and CAD prevalence.

		$\mu\text{g Ab Eq/mL Plasma}$
CAD	Unadjusted OR	12.45 (9.14-16.97) *
	Adjusted OR (1)	12.43 (7.57-20.42) *
	Adjusted OR (2)	14.18 (8.02-25.06) *

Results of fitting one univariate and two multivariable logistic regression models treating analyte (immunoglobulin against 3-nitrotyrosine) as a continuous variable. Shown are adjusted odds ratio and 95%CI for prevalent CAD per standard deviation change in log-transformed analyte measured.

Model 1: adjusted for traditional risk factors include age, gender, systolic blood pressure, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, smoking and diabetes.

Model 2: adjusted for traditional risk factors include age, gender, systolic blood pressure, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, smoking, diabetes, history of MI, aspirin, ACE, statin, beta-blockers.

\* P<0.001.