

# Atherosclerosis

## Aging-induced isoDGR-modified fibronectin activates monocytic and endothelial cells to promote atherosclerosis --Manuscript Draft--

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<b>Abstract:</b>	<p><b>Background and Aims</b> Aging is the primary risk factor for cardiovascular disease (CVD), but the mechanisms underlying age-linked atherosclerosis remain unclear. We previously observed that long-lived vascular matrix proteins can acquire 'gain-of-function' isoDGR motifs that might play a role in atherosclerotic pathology.</p> <p><b>Methods:</b> IsoDGR-specific mAb were generated and used for ELISA-based measurement of motif levels in plasma samples from patients with coronary artery diseases (CAD) and non-CAD controls. Functional consequences of isoDGR accumulation in age-damaged fibronectin were determined by bioassay for capacity to activate monocytes, macrophages, and endothelial cells (signalling activity, pro-inflammatory cytokine expression, and recruitment/adhesion potential). Mice deficient in the isoDGR repair enzyme PCMT1 were used to assess motif distribution and macrophage localisation in vivo .</p>

**Results:**

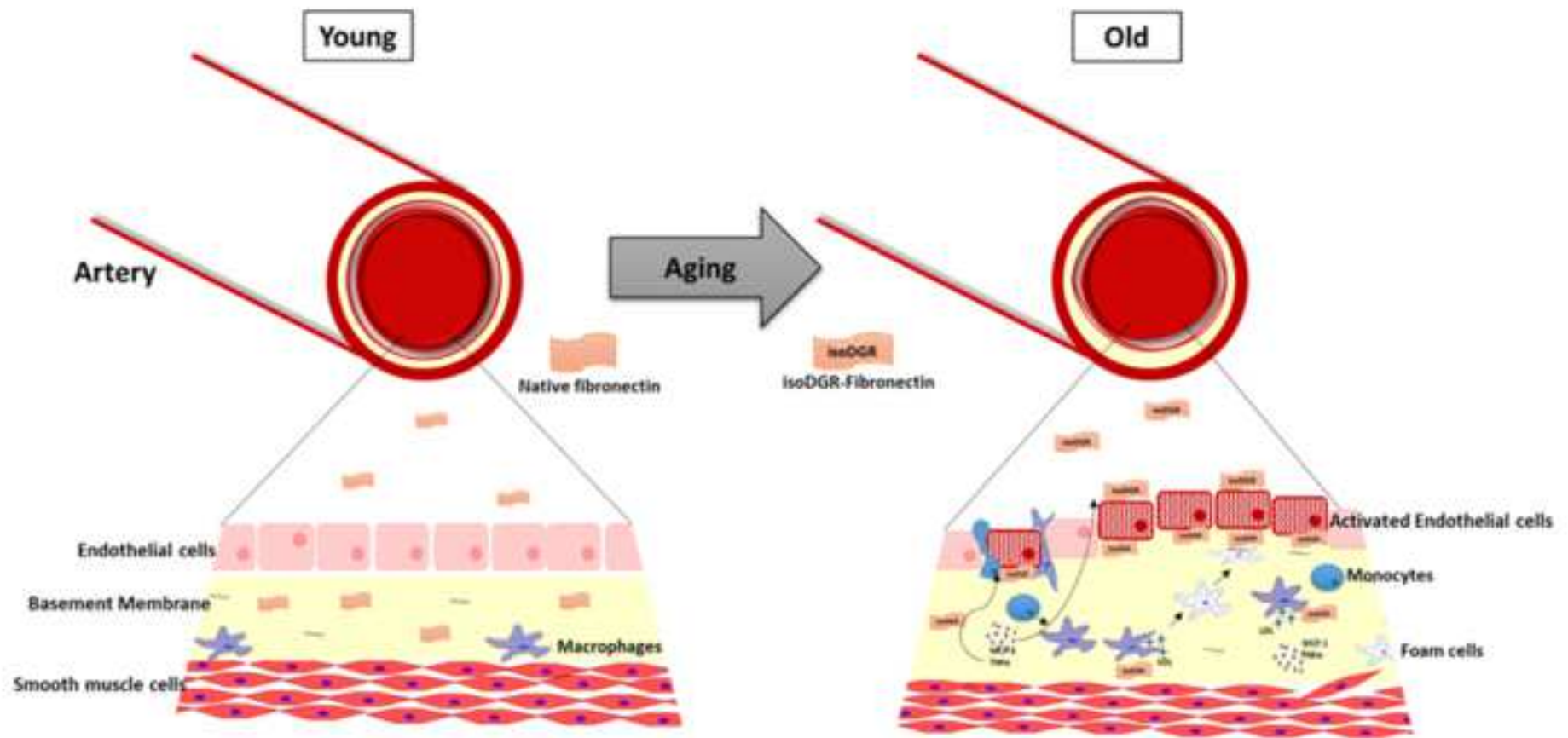
IsoDGR-modified fibronectin and fibrinogen levels in patient plasma were significantly enhanced in CAD and further associated with smoking status. Functional assays demonstrated that isoDGR-modified fibronectin activated both monocytes and macrophages via integrin receptor 'outside in' signalling, triggering an ERK:AP-1 cascade and expression of pro-inflammatory cytokines MCP-1 and TNF $\alpha$  to drive additional recruitment of circulating leukocytes. IsoDGR-modified fibronectin also induced endothelial cell expression of integrin b1 to further enhance cellular adhesion and matrix deposition. Analysis of murine aortic tissues confirmed accumulation of isoDGR-modified proteins co-localised with CD68+ macrophages *in vivo* .

**Conclusions:**

Age-damaged fibronectin features isoDGR motifs that increase binding to integrins on the surface of monocytes, macrophages, and endothelial cells. Subsequent activation of 'outside-in' signalling elicits a range of potent cytokines and chemokines that drive additional leukocyte recruitment to the developing atherosclerotic matrix.

**Highlights**

- IsoDGR-modified plasma proteins are associated with CAD.
- IsoDGR binding to integrin receptors on monocytes, macrophage, and endothelial cells promotes 'outside-in' signalling, monocyte infiltration, and endothelial binding.
- IsoDGR-modified fibronectin may initiate vascular inflammation in atherosclerotic CVD.



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**Aging-induced isoDGR-modified fibronectin activates monocytic and endothelial cells to promote atherosclerosis**

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**Short Title:** isoDGR-modified fibronectin promotes atherosclerosis

**Key words:** Atherosclerosis, vascular inflammation, fibronectin, isoDGR motif, integrin, deamidation

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## Abstract

### Background and Aims

Aging is the primary risk factor for cardiovascular disease (CVD), but the mechanisms underlying age-linked atherosclerosis remain unclear. We previously observed that long-lived vascular matrix proteins can acquire ‘gain-of-function’ isoDGR motifs that might play a role in atherosclerotic pathology.

### Methods:

IsoDGR-specific mAb were generated and used for ELISA-based measurement of motif levels in plasma samples from patients with coronary artery diseases (CAD) and non-CAD controls. Functional consequences of isoDGR accumulation in age-damaged fibronectin were determined by bioassay for capacity to activate monocytes, macrophages, and endothelial cells (signalling activity, pro-inflammatory cytokine expression, and recruitment/adhesion potential). Mice deficient in the isoDGR repair enzyme PCMT1 were used to assess motif distribution and macrophage localisation *in vivo*.

### Results:

IsoDGR-modified fibronectin and fibrinogen levels in patient plasma were significantly enhanced in CAD and further associated with smoking status. Functional assays demonstrated that isoDGR-modified fibronectin activated both monocytes and macrophages via integrin receptor ‘outside in’ signalling, triggering an ERK:AP-1 cascade and expression of pro-inflammatory cytokines MCP-1 and TNF $\alpha$  to drive additional recruitment of circulating leukocytes. IsoDGR-modified fibronectin also induced endothelial cell expression of integrin  $\beta$ 1 to further enhance cellular adhesion and matrix deposition. Analysis of murine aortic tissues confirmed accumulation of isoDGR-modified proteins co-localised with CD68+ macrophages *in vivo*.

### Conclusions:

Age-damaged fibronectin features isoDGR motifs that increase binding to integrins on the surface of monocytes, macrophages, and endothelial cells. Subsequent activation of ‘outside-in’ signalling elicits a range of potent cytokines and chemokines that drive additional leukocyte recruitment to the developing atherosclerotic matrix.

## Introduction

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2 Despite recent advances in therapeutic and preventive strategies, cardiovascular disease (CVD) remains  
3 a devastating disorder associated with extremely high morbidity and mortality across the world<sup>1</sup>.  
4 Previous research has now clearly demonstrated that aging is the primary risk factor for this complex,  
5 multifactorial disorder. Early events in CVD pathogenesis include endothelial dysfunction, vascular  
6 inflammation, and atherosclerotic plaque formation, ultimately leading to luminal narrowing and  
7 thrombotic occlusion of blood vessels<sup>2,3</sup>. While these key processes have now been investigated in detail,  
8 it remains unclear how tissue aging initiates the events required for later progression to overt pathology.  
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14 We recently reported that extensive deamidation of the amino acid sequence NGR (Asn-Gly-Arg) in  
15 extracellular matrix (ECM) proteins results in ‘gain-of-function’ conformational switching to isoDGR  
16 (isoAsp-Gly-Arg) motifs<sup>4-7</sup> that can mimic integrin-binding RGD ligands<sup>8-10</sup>. Atypical isoDGR  
17 modifications have previously been detected in fibronectin, laminin, tenascin C, and several other ECM  
18 proteins derived from human carotid plaque tissues<sup>4-7</sup>, suggesting that these molecules may be capable  
19 of enhancing leukocyte binding to the atherosclerotic matrix. Long-lived ECM components are well  
20 recognized to accumulate degenerative protein modifications (DPMs) including spontaneous  
21 deamidation<sup>11-17</sup>, under the influence of microenvironmental stresses, flanking amino acid sequences,  
22 and genetic factors<sup>13,17</sup>. DPMs are typically associated with loss of protein function and have long been  
23 regarded as critical events in human aging and degenerative disorders, but recent data have suggested  
24 that DPMs may also induce ‘gain of function’ structural changes that could play equally important roles  
25 in human pathology<sup>4-10</sup>. While age is the largest risk factor for many human chronic diseases including  
26 CVD, the mechanisms by which advancing age promotes atherosclerosis and CVD have remained  
27 obscure and the therapeutic opportunities these may provide are currently unknown. Having previously  
28 observed that long-lived ECM proteins undergo age-dependent spontaneous deamidation to generate  
29 isoDGR motifs, we postulate that age-related structural damage to key vascular proteins may represent  
30 the ‘missing link’ in common pathologies affecting the elderly. In particular, we hypothesize that  
31 atypical isoDGR:integrin interactions may serve as a key early trigger for endothelial dysfunction,  
32 macrophage activation, monocyte recruitment, and vascular inflammation leading to atherosclerotic  
33 plaque development.  
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50 Fibronectin is among the first matrix proteins deposited at sites of atherosclerosis<sup>18,19</sup>. Human  
51 fibronectin features a RGD integrin binding motif, as well as 6 NGR motifs that can be modified by  
52 spontaneous asparagine deamidation to isoDGR. Indeed, we have previously detected 4 characteristic  
53 isoDGR motifs in fibronectin derived from human carotid atherosclerotic plaques (at residues 263-265,  
54 367-369, 501-503 and 1432-1434)<sup>7</sup>. In the current study, we generated hybridoma cells that produce  
55 isoDGR-specific mAb and aimed to determine the effects of age-damaged fibronectin on monocyte and  
56 macrophage function in the early stages of CVD pathogenesis. We observed that interaction of isoDGR  
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1 motifs with integrins on the surface of monocytes and macrophages triggered an ERK:AP-1 signalling  
2 cascade that induced expression of a range of potent chemotactic mediators that promoted further  
3 leukocyte infiltration of the assembling atherosclerotic matrix. Biochemical and functional assays also  
4 demonstrated that isoDGR-modified FN promoted endothelial activation via expression of integrin  $\beta$ 1,  
5 which enhanced adhesion of blood leukocytes and facilitated protein deposition resembling intimal  
6 thickening in atherosclerosis. Crucially, administration of isoDGR-specific mAb was sufficiently to  
7 potently inhibit all of the pathological features assessed *in vitro*, suggesting that antibody blockade of  
8 this structure may represent a viable therapeutic option. Consistent with *in-vitro* results, we observed  
9 *in-vivo* accumulation of isoDGR-modified proteins co-localised with CD68+ macrophages in aortic  
10 tissues of mouse model. In addition, we detected significant enrichment of isoDGR motifs in plasma  
11 fibronectin from CVD patients analysed *ex vivo*, indicating isoDGR may be a predictive biomarker of  
12 CVD.  
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## 22 **Materials and methods**

### 23 ***Patients and clinical samples***

24 Study participants were recruited from patients undergoing coronary artery bypass grafting (CABG) or  
25 cardiac computed tomographic angiography (CCTA) at the Department of Cardiac, Thoracic and  
26 Vascular Surgery at the National University Heart Centre, National University Health System (NUHS)  
27 or the Department of Cardiology at Tan Tock Seng Hospital (TTSH). The study was approved by the  
28 institutional review boards of NTU (IRB-2017-01-013), NUHS (IRB-NUH-2009-0073) and TTSH  
29 (TTSH-2013-00930). Experimental procedures complied with the tenets of the Declaration of Helsinki.  
30 Informed written consent was obtained from each subject prior to the inclusion in the study. For detailed  
31 demographic information and clinical history see; Table 1, Supplementary Data 1 (S.Tables 1 and 2),  
32 Fig. 1. Patients who were diagnosed with atherosclerotic coronary artery disease (CAD) by  
33 angiography and underwent CABG were included (n=25) and age-matched control subjects were  
34 selected from patients who were assessed by angiography/CCTA on clinical suspicion but found to be  
35 free of CAD (n=25). All blood samples were collected prior to angiography, CCTA or CABG.  
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### 46 ***Cell lines and culture methods***

47 Cell culture media and supplements were obtained from Life Technologies Holdings Pte Ltd. (Grand  
48 Island, NY, USA) unless otherwise specified. Human monocytic cell lines THP-1 and U937, and murine  
49 macrophage cell line RAW264.7 were cultured in RPMI-1640 medium supplemented with 10% fetal  
50 bovine serum (FBS) and 1% penicillin/streptomycin (P/S) at 37°C in 5% CO<sub>2</sub>. Human umbilical vein  
51 endothelial cells (HUVECs) were cultured in DMEM containing 10% FBS and 1% P/S. All cell lines  
52 were tested for mycoplasma contamination by PCR according to published methods<sup>20</sup>. Macrophages  
53 were differentiated from U937 and THP-1 cells by incubating the cells with 100ng/ml Phorbol 12-  
54 myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO, USA) for 1h in RPMI-1640 medium  
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1 containing 1% FBS. The RAW264.7 cells, monocytic U937/THP-1 cells and derivative macrophages  
2 were seeded onto fibronectin-coated 24 well plates. To assess the role of isoDGR:integrin receptor  
3 interactions in CVD, cell pellets were collected for western blot and RT-PCR analysis to determine  
4 'outside-in' signalling activity and cytokine expression. Cell-conditioned medium was also assayed for  
5 pro-inflammatory cytokine levels. In some experiments, isoDGR:integrin interactions was blocked by  
6 addition of isoDGR-specific mAb (10µg/ml).  
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### 10 11 ***Cytokine multiplex bead assay.***

12 The LEGENDplex™ mouse inflammation panel (Biolegend, San Diego, CA) was used to measure 13  
14 different cytokines (IL-23, IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-17A, IL-23, IL-27, MCP-1, IFN-β,  
15 IFN-γ, TNF-α, and GM-CSF) in cell culture supernatants after 48h incubation (assessed by LSRII flow  
16 cytometer according to the manufacturer's protocol).  
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### 20 21 ***Secreted alkaline phosphatase (SEAP) reporter assay***

22 U937 cells were reverse-transfected with 50ng AP1-SEAP or NFκB-SEAP vector, then seeded onto  
23 native or deamidated fibronectin-coated 96-well plates and incubated for 24h. Culture supernatants  
24 were collected, heated at 65°C for 30min, then assayed for alkaline phosphatase activity as follows;  
25 30µl of supernatant was incubated with 120µl assay buffer for 5min, after which 1:20 diluted CSPD  
26 substrate was added and the samples read on a TECAN microplate reader (Maennedorf, Switzerland).  
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### 32 33 ***Transwell migration assay***

34 Native or deamidated fibronectin were coated onto 96-well plates (2.5µg/100µl volume per well) via  
35 overnight incubation at 4°C. Control wells were prepared with 1% bovine serum albumin (BSA) in PBS  
36 and transmigration assays were carried out using n=5 technical replicates. Briefly, U937 cells were  
37 primed with PMA (100ng/ml) for 1h at 37°C then re-suspended in 1% FBS-RPMI medium and seeded  
38 onto fibronectin-coated plates at a density of 1 x 10<sup>5</sup> cells/well for 6h. Thereafter, 4 x 10<sup>4</sup> CFSE-labelled,  
39 non-activated U937 cells were added to matrigel-coated inserts and incubated for a further 24h at 37°C.  
40 Cell transmigration in response to molecules secreted by fibronectin-bound monocytes was monitored  
41 using fluorescence microscopy and quantified by crystal violet staining. IsoDGR-specific mAb was  
42 added to the deamidated fibronectin condition to determine motif specificity of these effects.  
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### 51 52 ***Fibronectin fibrillogenesis and DOC solubility assay***

53 Native and deamidated fibronectin were labelled with Alexa488 dye and used for exogenous plasma  
54 fibronectin assembly assays. Briefly, HUVECs were seeded onto coverslips and allowed to adhere and  
55 grow to 90-100% confluency. Subsequently, cells were treated with either native or deamidated FN  
56 (25µg/ml) in 2% FBS-DMEM media containing PMA (100ng/ml) and incubated for the indicated times  
57 at 37°C with or without steady flow (100rpm). Fibronectin deposition and/or matrix assembly were  
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1 monitored using fluorescence microscopy and subsequently processed for sodium deoxycholate (DOC)  
2 solubility assays as previously described<sup>21</sup>. Briefly, HUVECs were put on ice and lysed with cold DOC  
3 lysis buffer (2% DOC; 20mM Tris-HCl, pH 8.8; 2mM PMSF; 2mM EDTA; 2mM iodoacetic acid;  
4 2mM N-ethylmaleimide). Cells were lysed using a 26G syringe needle and immediately centrifuged at  
5 15,000rpm for 15min at 4°C. The supernatant was collected as DOC-soluble fraction while the pellet  
6 was washed with DOC buffer and then solubilized in 25µl SDS buffer (1% SDS; 20mM Tris-HCl, pH  
7 8.8; 2mM PMSF; 2mM EDTA; 2mM iodoacetic acid; 2mM N-ethylmaleimide). DOC-soluble and  
8 DOC-insoluble fractions were subsequently resolved by SDS-PAGE using 7.5% polyacrylamide gels,  
9 then transferred onto nitrocellulose membranes and immunoblotted with anti-fibronectin mAb.  
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### 16 ***Murine model***

17 Mice deficient in the deamidation repair enzyme *PCMT1* (C57BL/6 background) were obtained from  
18 The Jackson Laboratory (*PCMT1*<sup>-/-</sup> mice die prematurely, so *PCMT1*<sup>+/-</sup> animals were used in all  
19 experiments)<sup>22</sup>. Mouse genotype was confirmed by PCR using the following primers: oIMR1080:CGG  
20 CTG CAT ACG CTT GAT C, oIMR1081:CGA CAA GAC CGG CTT CCA T, oIMR1544:CAC GTG  
21 GGC TCC AGC ATT, oIMR3580:TCA CCA GTC ATT TCT GCC TTT G. Mice were maintained on  
22 normal chow diets and housed with regular light/dark cycles.  
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### 30 ***Immunostaining of aorta sections***

31 Aorta from 8-month-old WT and *PCMT1*<sup>+/-</sup> mice were collected and fixed with 4% paraformaldehyde  
32 at 4°C for 24h. The aorta tissues were washed with PBS and supplemented with 15% sucrose then 30%  
33 sucrose over 1 day. Aortas were embedded in OCT compound with dry ice. A Leica CM3060S Cryostat  
34 was used to prepare 10µm cross sections of aorta that were mounted on Fisherbrand Superfrost plus  
35 Microscope slides. The slides were kept in warm PBS for 20min to remove OCT before being  
36 permeabilized and treated for 1h with blocking buffer (2.5% normal Goat serum, 1% BSA in 0.5% PBST)  
37 prior to addition of primary antibodies (isoDGR [1:200] and CD68 [1:200]) overnight at 4 °C. The  
38 slides were then incubated with secondary antibodies conjugated to AlexaFluor 488 and 594 for 1h at  
39 RT. DAPI staining for 30min was used to visualize cell nuclei. After staining, the slides were washed  
40 with 1X PBS and mounted with aqueous mounting media. Images were acquired using a Zeiss LSM710  
41 confocal microscope.  
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### 51 ***Statistical analysis***

52 Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego,  
53 CA). Differences between groups were assessed either by one-way ANOVA or Student's t-test (p<0.05  
54 was considered significant).  
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## Results

### **IsoDGR motifs are increased in blood plasma from patients with coronary artery disease (CAD)**

Deamidation of NGR (Asn-Gly-Arg) amino acid sequences in extracellular matrix (ECM) proteins creates ‘gain-of-function’ isoDGR motifs (isoAsp-Gly-Arg) implicated in atherosclerotic plaque formation, so we developed a specific monoclonal antibody against isoDGR to enable analysis of this structure in human patient samples (Fig. 1). In previous analyses of human carotid plaque tissues, we identified high levels of deamidation in core proteins fibronectin (FN) and fibrinogen (FBG) which are known to play critical roles in CVD<sup>23,24</sup>. To determine isoDGR levels in blood plasma from CVD patients, we collected samples from n=25 CAD patients immediately prior to CABG as well as n=25 control subjects (confirmed to be free of CAD by angiography or CCTA; Table 1, Supplementary Data 1 (S.Tables 1 and 2)). An isoDGR-specific mAb was then used to measure total isoDGR-modified plasma proteins by ELISA (S.Fig. 1).

These analyses showed that levels of total isoDGR-modified plasma proteins were significantly increased in CAD relative to control patients ( $p < 0.0001$ ) (Fig. 1b). Similarly, when we specifically measured levels of native versus modified FN and FBG in blood plasma, we observed that isoDGR-modified variants of these proteins were significantly higher in CAD (Fig. 1c and d), even though total concentrations of FN and FBG were comparable between groups (Fig. 1e and f). IsoDGR formation is predominantly a consequence of aging, but it is unclear whether accumulation of this motif can also be influenced by other CVD risk factors. We therefore assessed total isoDGR levels in CAD and CTRL subjects were correlated with clinical hypertension (S.Fig. 2a), smoking status (S.Fig. 2b), or hyperlipidemia/statin therapy (S.Fig. 2c). These analyses revealed a significant association of smoking status with higher levels of plasma isoDGR in CAD patients that warrants further investigation in future.

### **isoDGR formation in fibronectin induces activation of integrin ‘outside-in’ signaling pathways**

Human fibronectin features a RGD integrin-binding motif and 6 potential isoDGR motifs that may significantly impact human health in advancing age. In previous work, we reported that fibronectin in carotid plaques accumulates high levels of isoDGR in 4 of 6 possible motifs, and these motifs are capable of enhancing monocyte adhesion to fibronectin-coated plates via interaction with cell-surface integrins<sup>7</sup>. In the current study we sought to determine the biological consequences of integrin-mediated immune cell adhesion to isoDGR-modified fibronectin. To do this, we incubated the native protein in mild alkaline condition (pH 8.0) to promote spontaneous deamidation of the NGR sequence. The deamidation of Asn amino acid residue via a succinimide intermediate generates a mixture of D- or L-Asp, and D- and L-isoAsp products. The reaction mixture was therefore separated and characterized by RPLC-MS/MS using a C18 column and ERLIC-MS/MS using WAX column<sup>25-28</sup>. Native fibronectin displayed little evidence of deamidation (Supplementary Data 2A), whereas 3 isoDGR motifs that were previously identified in human carotid atherosclerotic plaque were detected in the treated FN protein

1 (Supplementary Data 2 to 6). The reaction should generate the D-/L- stereoisomers that were not  
2 distinguished by our non-chiral RPLC and ERLIC separation, likely because typical D-/L-  
3 stereoisomers require chiral chromatography for separation. However, peptides featuring NGR, D-/L-  
4 DGR and D-/L-isoDGR motifs derived from the same precursor sequence could be well-separated with  
5 ERLIC-MS/MS peptides following the expected elution order (-NGR- < -DGR- < -isoDGR-), thereby  
6 facilitating estimation of isoDGR motif yields (DGR : isoDGR, ~1 : 3). Based on label-free quantitation  
7 using XIC of the LC-MS/MS data, our fibronectin deamidation method induced isoDGR motifs at  
8 residues 263-265 (~10%), 501-503 (3%), and 1432-1434 (8%). The isoDGR motif at residues 263-265  
9 is generated as the major product located on fibronectin subunit I, facing outwards from the main body  
10 of the molecule (see 3D structure in Fig. 2A and B), suggesting that this *de novo* structure is likely to  
11 be readily accessible for integrin binding.  
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20 To assess the biological consequence of fibronectin deamidation, murine RAW macrophages were  
21 cultured in 24-well plates containing either native or deamidated fibronectin for 24h incubation then  
22 subjected to total protein extraction and western blot analysis. As shown in Fig. 2c, macrophages  
23 displayed high basal levels of integrin  $\beta$ 3 expression that were increased upon adhesion to deamidated  
24 fibronectin, whereas AKT phosphorylation was unaltered by fibronectin binding state. Instead,  
25 interaction of macrophage integrins with deamidated fibronectin appeared to activate the alternative  
26 MAPK-ERK signaling pathway as indicated by phosphorylation of ERK1/2. The specific requirement  
27 for isoDGR-modified fibronectin to trigger ‘outside-in’ signaling was confirmed by addition of an  
28 isoDGR neutralizing antibody that blocked integrin  $\beta$ 3 upregulation and ERK1/2 activity (Fig. 2c).  
29 Similar observations were confirmed in human macrophages generated from PMA-primed U937  
30 monocytes (Fig. 3d). To further investigate the effects of NGR-to-isoDGR molecular switching on  
31 downstream pathways of integrin ‘outside-in’ signalling, we next used reporter constructs containing  
32 the secreted alkaline phosphatase (SEAP) gene under the control of either AP-1 or NF- $\kappa$ B promoters to  
33 quantify pathway activation. For this assay, we used human U937 cells (Fig. 2d). Monocytes binding  
34 to deamidated fibronectin displayed a significant increase in AP-1-induced SEAP activity compared to  
35 native protein-exposed cells, indicating that isoDGR motifs can promote signal transduction through  
36 this axis and potentially upregulate downstream gene targets of AP-1. Intriguingly, isoDGR-triggered  
37 integrin signalling appeared independent of NF- $\kappa$ B which was not activated in our assays despite this  
38 pathway playing a central role in regulating inflammation<sup>29</sup>.  
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#### 54 **NGR switching to isoDGR increases expression of chemotactic mediators**

55 Since AP-1 transcription factors regulate cytokine gene expression in response to a variety of external  
56 signals,<sup>30,31</sup> we next examined mRNA expression levels of pro-inflammatory mediators after 24h  
57 exposure of RAW (Fig. 3a) or PMA-primed U937 cells (SFig. 3) to either native or deamidated  
58 fibronectin. Fig. 3a displays expression levels of various cytokine/chemokine genes in murine  
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macrophages, indicating that exposure to deamidated fibronectin induced substantial upregulation of several chemotactic molecules including monocyte chemoattractant protein (MCP)-1, GM-CSF, CCL4, and INF- $\beta$ , as well as the metalloprotease enzyme MMP9 and associated pro-inflammatory cytokine TNF $\alpha$ . Since secretion of chemokine(s) by tissue-resident macrophages promotes monocyte recruitment to the vessel walls, we next measured 13 pro-inflammatory mediators in the cell-conditioned medium using a multiplex bead array. These analyses showed that levels of MCP-1 and TNF $\alpha$  in the conditioned medium were increased by >4-fold and >3-fold respectively upon macrophage exposure to deamidated fibronectin (Fig. 3b). Accordingly, macrophage secretion of MCP-1 and TNF $\alpha$  were blocked by addition of neutralizing isoDGR-specific mAb, indicating that these events depended on isoDGR motifs.

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Since macrophage adhesion to isoDGR-modified fibronectin induced marked upregulation of pro-inflammatory cytokine genes and high secretion of MCP-1 and TNF $\alpha$ , we next hypothesized that altered patterns of cytokine expression may be a key event promoting further leukocyte accumulation and establishing a ‘positive feedback loop’ that drives pathology in CVD. To test this, we established a Transwell migration assay using PMA-primed U937 cells that were seeded into the bottom chambers of culture plates that were coated with either native or deamidated fibronectin (or uncoated control wells). After 6h incubation, unstimulated monocytes pre-labelled with CFSE were seeded into the Matrigel-coated upper chambers and their migration patterns were monitored by fluorescence microscopy. As shown in Fig. 3c, U937 cells exposed to deamidated fibronectin displayed >2-fold more transmigration than did cells exposed to either native fibronectin or uncoated control wells. The addition of isoDGR neutralizing antibody significantly reduced/blocked transmigration in these assays (SFig. 4). Together, these data confirmed that macrophage adhesion to isoDGR-modified fibronectin triggers ‘outside in’ signalling to promote the release of soluble factors that promote further leukocyte recruitment to the age-damaged matrix and likely represents a key early event in atherosclerotic plaque formation/progression. Indeed, subsequent differentiation of infiltrated monocytes into intima-resident macrophages is another key step in atherosclerosis, and U937 cells exposed to deamidated fibronectin displayed significantly increased expression of macrophage-associated integrin  $\beta$ 3 and pERK42/44 activity (Fig 3d, PMA- data).

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The above *in-vitro* functional assays strongly suggested that isoDGR-modified fibronectin is a critical mediator of vascular inflammation via interaction with resident macrophages in early stage atherosclerotic CVD. We therefore sought to determine whether this process can also be enhanced by isoDGR-modified proteins *in-vivo* using a mouse model deficient in the deamidation repair enzyme PIMT (L-isoaspartyl methyltransferase, or PIMT/PCMT1)<sup>22</sup>. Since *PCMT*<sup>-/-</sup> mouse died prematurely, we used 8-month old WT and *PCMT*<sup>Het</sup> mice to study the accumulation of isoDGR motifs and CD68+ macrophages in aorta tissues over time. When assessed by immunofluorescent imaging, we observed

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marked accumulation of isoDGR motifs in both intima and adventitial layers of both WT and *PCMT*<sup>+/-</sup> mice, but more extensive accumulation of isoDGR-modified proteins with co-localised CD68<sup>+</sup> macrophages in aorta from transgenic animals (Fig 3e). Combined with our *in vitro* results, these *in vivo* data confirm an important role for isoDGR motifs in vascular inflammation and immune cell recruitment in the early phases of atherosclerosis.

### **Deamidated fibronectin activates endothelial cell adhesion via expression of integrin $\beta$ 1**

Activated endothelium expresses a series of  $\beta$ 1-containing integrin pairs that can mediate cell adhesion ( $\alpha$ 1 $\beta$ 1,  $\alpha$ 2 $\beta$ 1,  $\alpha$ 3 $\beta$ 1,  $\alpha$ 5 $\beta$ 1 and  $\alpha$ 6 $\beta$ 1)<sup>32</sup>, so we next sought to determine whether isoDGR-modified fibronectin could alter patterns of  $\beta$ 1. To test this, we added either native or deamidated fibronectin (25 $\mu$ g/ml) to PMA-primed human umbilical vein endothelial cells (HUVECs) for 24h and performed immunofluorescent staining for active integrin  $\beta$ 1 (using antibody clone HUTS-4 which recognizes epitopes in the 355–425 region of active  $\beta$ 1 subunits<sup>33</sup>). Levels of activated integrin  $\beta$ 1 were markedly increased on HUVECs exposed to deamidated fibronectin relative to those treated with native protein alone. Active integrin  $\beta$ 1 was also more clearly distributed to the plasma membrane in the presence of isoDGR-modified fibronectin (Fig. 4a, 4b). To further elucidate possible activation signals triggered by modified fibronectin, we next exposed PMA-primed HUVECs to a range of synthetic peptides containing either NGR, RGD, or isoDGR motifs for 24h before repeating the immunofluorescent analyses. Unlike peptides containing NGR or RGD sequences, ligands that featured an isoDGR motif potently induced integrin  $\beta$ 1 activation and distribution to the membrane region of PMA-primed HUVECs (Fig 4c). However, HUVECs routinely failed to react to fibronectin in the absence of PMA priming, suggesting that basal integrin activation is prerequisite for isoDGR motifs to influence endothelial  $\beta$ 1 function.

### **Fibronectin NGR-to-isoDGR transition facilitates fibrillogenesis and insoluble matrix formation**

Fibronectin:integrin interactions initiate step-wise conformational changes in fibronectin: fibronectin interactions on cell surfaces that support conversion to insoluble fibrils / fibrillogenesis<sup>34,35</sup>. Since our data suggested that deamidated fibronectin can enhance activation of integrins - which are the principal fibronectin receptors in endothelial cells - we next examined whether protein deamidation was able to modify normal processes of polymerization, accumulation, and fibril formation that may lead to intima thickening. To do this, we incubated PMA-primed HUVECs for variable duration with either native or deamidated plasma fibronectin (25 $\mu$ g/ml) that had been pre-labelled with AlexaFluor488 dye. When visualized by microscopy, we rarely observed any deposition of native fibronectin on HUVECs after 6h exposure, and protein aggregation could only be detected after an extended 18h incubation period (Fig. 4d). In contrast, deposition of deamidated fibronectin was clearly visible within just 6h and protein aggregates on HUVECs were greatly increased over time. Since vascular endothelial cells are exposed

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to constant mechanical force *in vivo*,<sup>36</sup> we next investigated the dynamics of native versus deamidated fibronectin assembly on PMA-primed HUVECs subjected to continuous flow (CO<sub>2</sub> shaking incubator for 18h duration). Under these conditions, deposits of deamidated fibronectin converted to stretched and unfolded forms on endothelial cell surfaces, whereas native protein accumulated in greater quantities but failed to form extended fibrils, suggesting that deamidation of plasma fibronectin facilitates matrix assembly and formation of dense fibril networks (Fig. 4d). To further test whether isoDGR-containing fibronectin can accelerate the formation of stable and mature fibrillary networks, we next used a deoxycholate (DOC) detergent assay to assess how deamidated FN impacts matrix solubility, which is a critical determinant of overall tissue stability, rigidity, and shape<sup>37,38</sup>. Western blot analysis of proteins obtained by DOC buffer-extraction from HUVECs revealed a significant increase in insoluble fibronectin content among cells that had been exposed to isoDGR-containing variant relative to the unmodified protein (p=0.0185; Fig. 4e).

## Discussion

CVD is a major public health concern associated with an enormous socio-economic burden as both incidence and associated mortality continue to increase around the world<sup>39</sup>. Many CVD-related deaths could potentially be avoided by effective health management after early detection of key risk factors. While it has long been established that CVD is a multifactorial disorder, aging remains the single largest risk factor for this complex disease. Over 70 years ago, the landmark Framingham Heart Study defined major risk factors for CVD which is driven by a complex interplay of age, genetics, smoking, nutrition, obesity, and other factors<sup>40</sup>. Progress in understanding the pathological mechanisms at play have led directly to the development of current anti-platelet, anti-thrombotic, anti-hypertensive, anti-diabetic, and anti-lipid drug therapies. Unfortunately, the mechanisms by which advancing age promotes atherosclerosis and intravascular thrombosis have remained elusive and the therapeutic opportunities these may provide are currently unknown. In the current study, we therefore sought to identify critical age-linked mediator of early events in atherosclerotic CVD.

Numerous recent studies have shown that biomolecule damage by spontaneous chemical reactions (i.e. degenerative protein modifications; DPMs) as a major contributor to age-linked diseases<sup>13-16,41-44</sup>. We have previously reported that specific matrix proteins in carotid atherosclerotic plaque from CVD patients exhibit marked deamidation of NGR motifs<sup>5</sup>, thus facilitating increased monocyte binding to core matrix components<sup>7</sup>. In the current study, we generated a novel isoDGR-specific mAb enabling screening of blood plasma samples and functional studies of isoDGR biology in atherosclerotic CVD. To address the potential role of deamidated fibronectin in the vascular network and potential correlations with CVD pathology, we investigated effects of the isoDGR-modified protein on leukocytes and inflammation, as well as endothelial cell biology using a series of phenotypic and functional assays. In these experiments, isoDGR-modified fibronectin was consistently found to

1 promote macrophage activation and monocyte recruitment/infiltration. Macrophages and monocytes  
2 can bind to ECM via integrin interactions with RGD or isoDGR motifs<sup>7,10</sup>. Our data now indicate that  
3 isoDGR:integrin signalling is a specific feature of the atherosclerotic matrix that triggers differential  
4 patterns of gene expression in adherent leukocytes, and that this process can be potently inhibited by  
5 isoDGR-specific mAb. Indeed, MAPK/ERK-dependent signalling and upregulation of transcription  
6 factor AP-1 were induced only in macrophage and monocytic cells binding to deamidated fibronectin,  
7 resulting in amplification of several chemokine/cytokine genes and transmigration of additional  
8 immune cells towards the assembling matrix. While AP-1 signal transduction is known to be activated  
9 by MAPK/ERK activation in response to a variety of external cues<sup>30,45,46</sup>, the mechanism by which the  
10 isoDGR:integrin:ERK:AP-1 axis induces signals in CVD will require further investigation. Intriguingly,  
11 isoDGR potently triggered macrophage secretion of MCP-1 and TNF $\alpha$ . Chemoattractant MCP-1 is a  
12 crucial mediator of monocyte/macrophage recruitment to the endothelial basement membrane during  
13 atherosclerosis development<sup>47</sup>, with MCP-1 knock-out animals displaying significant decreases in both  
14 plaque size and progression<sup>47-49</sup>. Another upregulated mediator TNF- $\alpha$  stimulates endothelial  
15 production of adhesion molecules that promotes binding of circulating monocytes<sup>50</sup>. The above results  
16 clearly indicate that age-damaged fibronectin which features isoDGR motifs can enhance binding to  
17 blood leukocytes and stimulate production of pro-inflammatory mediators MCP-1 and TNF $\alpha$  that drive  
18 recruitment of additional monocytic cells from the circulation. In summary, our data suggest that  
19 molecular switching from NGR to isoDGR motifs facilitates fibronectin binding to integrins on the  
20 surface of macrophages and monocytes leading to ‘outside-in’ signalling and induction of a range of  
21 potent pro-inflammatory mediators. Together, these events drive further leukocyte recruitment and  
22 accumulation in the artery wall, likely contributing to early pathological events in atherosclerotic CVD.  
23 Moreover, isoDGR motifs were also found to increase endothelial adhesion by upregulation of the  
24 integrin  $\beta$ 1 subunit. Furthermore, isoDGR-modified fibronectin interacted with endothelial cell layers  
25 where the modified protein accelerated formation of stable, insoluble matrix that may contribute to  
26 intima thickening. These data suggest that fibronectin ‘aging’ generates isoDGR motifs that activate  
27 immune cells and endothelial cells via integrin receptor interactions.

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47 Taken together, our results demonstrated that aged fibronectin which features isoDGR might initiate a  
48 vicious cycle driving the pathology of atherosclerotic CVD. Age-induced isoDGR activated resident  
49 macrophages to express/secrete proinflammatory cytokines and chemokines like MCP-1, thereby  
50 helping recruit yet more blood monocytes into the vascular wall. Moreover, both isoDGR stimulated  
51 proinflammatory cytokines (TNF $\alpha$ ) and isoDGR itself can activate endothelial cells to enhance  
52 adhesion to circulating leukocytes. Upon interaction with isoDGR-modified fibronectin, infiltrated  
53 monocytes began differentiating into macrophages that are known to acquire LDL and form foam cells.  
54 This vicious cycle may repeat and amplify atherosclerotic plaque development and progression (Fig.5).



1 Our ELISA results revealed that CAD is associated with increased plasma levels of isoDGR-modified  
2 fibronectin and fibrinogen which are known to be critical mediators of arterial thrombosis. Moreover,  
3 the phenotypic and functional experiments suggest that isoDGR formation may be a key early event in  
4 the pathogenesis of atherosclerotic CVD that may be detected and accurately quantified using an  
5 isoDGR-specific mAb ELISA assay. Similar approaches could potentially also be used to develop  
6 routine blood tests for early detection of atherosclerotic CVD, thereby facilitating timely intervention  
7 to reduce mortality (e.g. via primary prevention strategies targeting modifiable risk factors like  
8 smoking/cholesterol/diet, or medical management with statins and blood thinners etc). **However, further  
9 assessment of this possibility in larger cohorts and prospective studies will now be required since the  
10 control subjects used in the current study were limited to individuals with suspected CAD (despite  
11 subsequently diagnosis as non-CAD cases by angiography or CCTA).** Our data also suggest that  
12 blocking/clearing isoDGR-motifs from the vasculature may be therapeutic and/or prophylactic in CVD  
13 and possibly also other age-linked diseases. **Intriguingly, many ECM proteins with native 'RGD'  
14 integrin binding motif have 'NGR' sequence motifs susceptible to formation of isoDGR motifs during  
15 aging, and were detected in atherosclerotic plaque in our previous study<sup>4,7</sup>. Further investigation of  
16 these isoDGR-modified protein function and therapeutic targeting in both animal models and patient  
17 tissues should therefore be a priority for future studies.**

### 33 **Conflict of interest**

34 The authors declare no conflict of interest.

### 38 **Financial support**

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### 45 **Author contributions**

46 J.E.P. and G.J. performed most *in-vitro* experiments, data analysis and drafted the manuscript; K.P.  
47 performed animal experiments and data analysis; X.G. performed LC-MS/MS; S.C.N. performed  
48 ERLIC-MS/MS analysis; K.C.K, S.S.N, S.E.L. performed qPCR and WB experiments; W.M.  
49 performed MALDI-TOF-MS analysis; S.C.L., M.K.L., D.J.P., D.K., V.S, H.H.H and N.E.M.  
50 contributed clinical samples, reagents and revised the manuscript; S.K.S. conceived and supervised the  
51 project and wrote the manuscript. All co-authors contributed to the revision of the manuscript.

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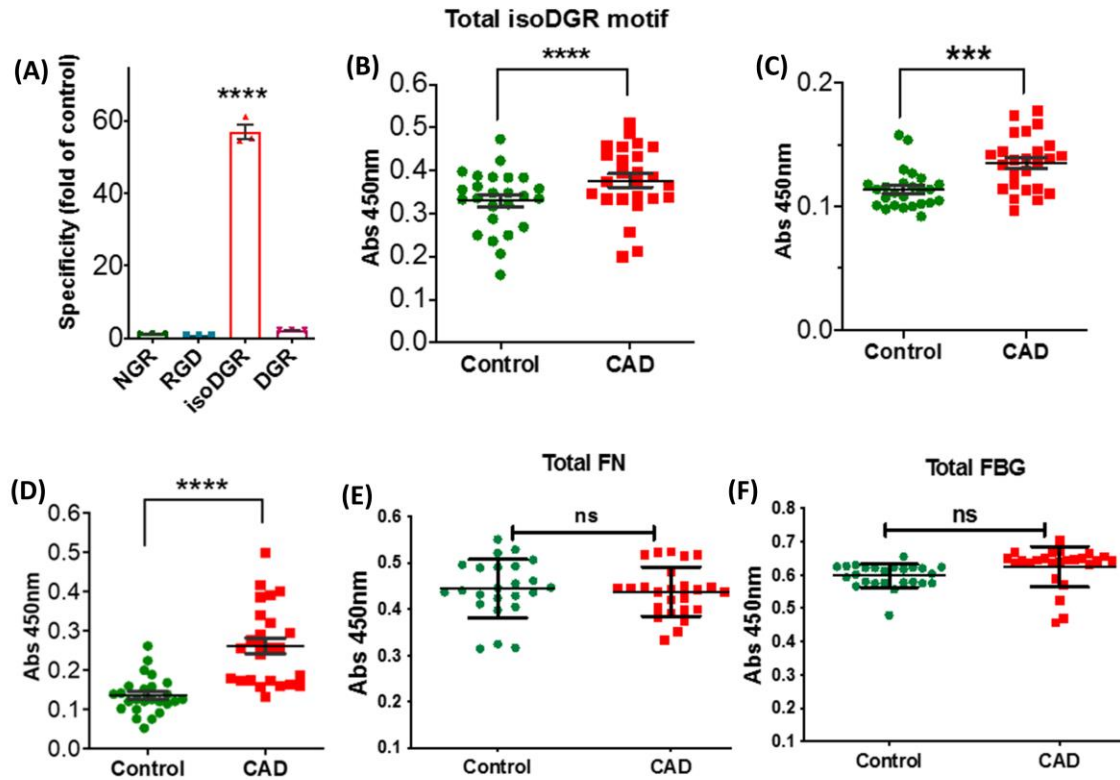
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## Figures and Tables

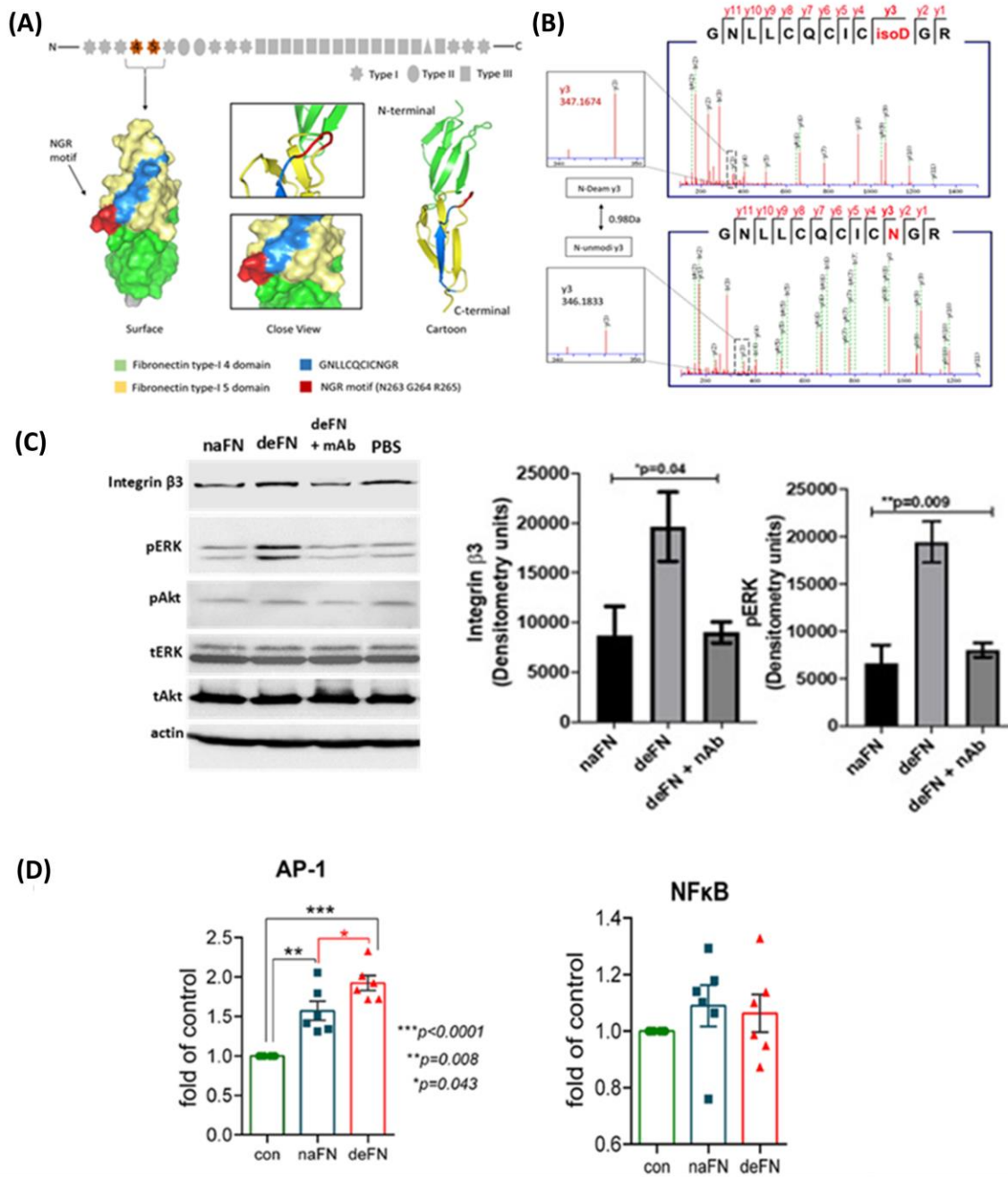
**Table 1.** Demographic summary of clinical cohorts.

	<b>Ischemic Heart Disease</b>	<b>Control</b>
	<b>(IHD)</b>	<b>(Ctrl)</b>
	<b>(N=25)</b>	<b>(N=25)</b>
Age [Mean ( $\pm$ SD)]	63 ( $\pm$ 7.6)	54 ( $\pm$ 8.7)
Gender [N (%)]		
Male	18(72)	15 (60)
Female	7(28)	10(40)
Chinese [N (%)]	25 (100)	25(100)
Treatment [N (%)]		
CABG	25(100)	NA
Conservative	NA	25(100)
Hypertension [N (%)]		
Hypertension	19 (76)	5 (20)
No Hypertension	6(24)	20(80)
Hyperlipidemia [N (%)]		
Hyperlipidemia	25(100)	8 (32)
No Hyperlipidemia	NA	17(68)
Diabetes Mellitus [N (%)]	15 (60)	14 (70)
Lipid medication (statin therapy) [N (%)]	25(100)	10(40)
Smoking [N (%)]		
Smoking	9(36)	1(4)
No Smoking	14(56)	24(96)
Missing information	2(8)	NA



**Figure 1: IsoDGR detection in blood plasma from patients with coronary atherosclerosis**

(A) ELISA results using synthetic peptides indicated that mAb specifically binds peptides that contain an isoDGR motif (but not those containing unmodified NGR, RGD, or DGR sequences). (B) Measurement of total isoDGR levels in plasma samples from control subjects (n=25) or CAD patients (n=25) by indirect ELISA with isoDGR-specific mAb. (C) and (D) Sandwich ELISA analysis of isoDGR motif level in plasma FN (c) and FBG (d) in the same set of plasma samples. (E) and (F) Sandwich ELISA analysis of native/unmodified plasma FN (e) and FBG (f). ELISA assay was performed with three technical replicates (n=3). Statistical differences between groups were determined by unpaired T-test; \*\*\* $p=0.003$ , \*\*\*\* $p<0.0001$ , ns=no significant difference.



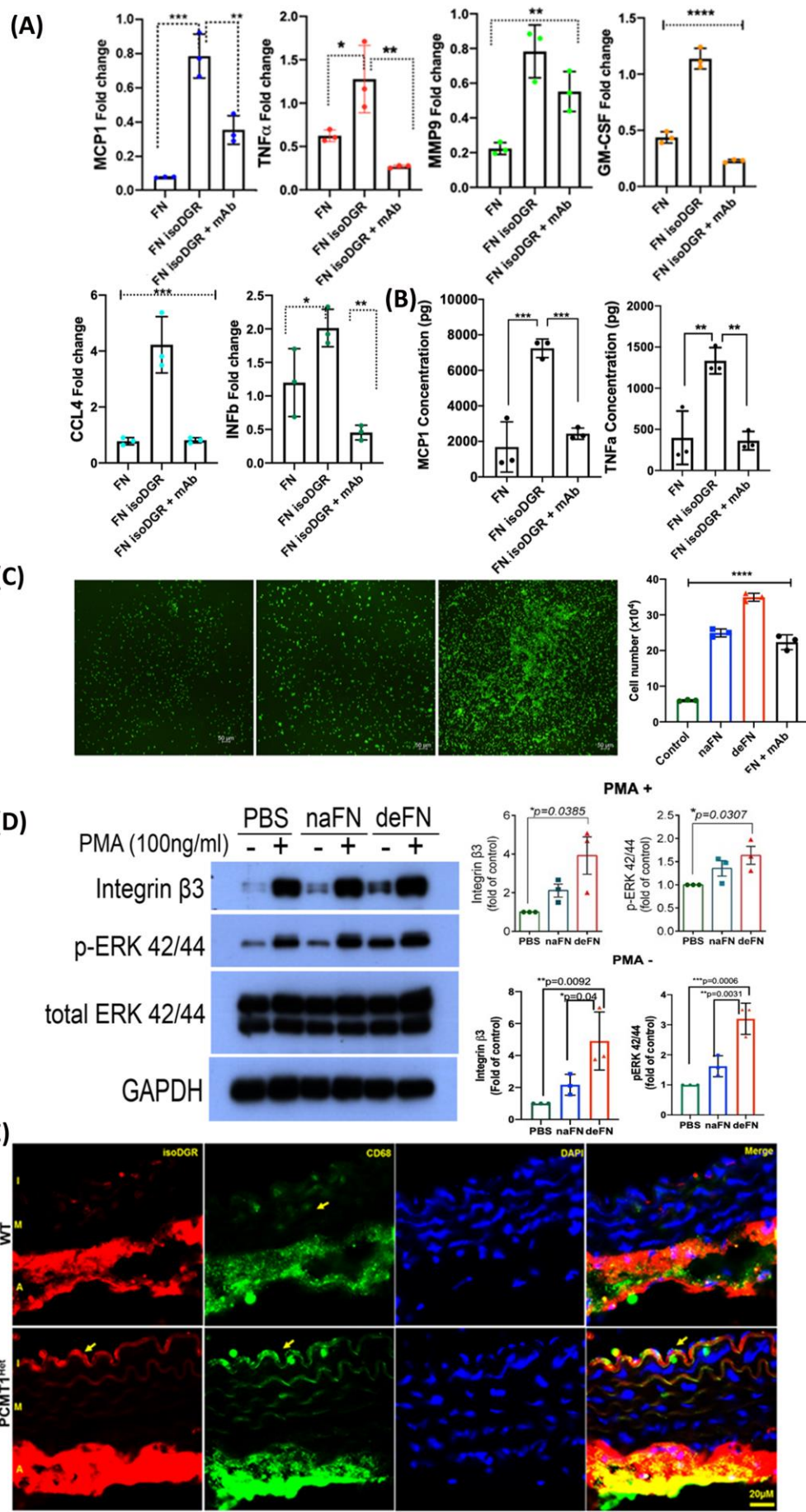
**Figure 2: IsoDGR-modified fibronectin activates macrophages via pERK**

(A) The NGR motif (red) is located within the type I domain and faces outwards from the main body of the molecule. (B) HCD mass spectra of peptide  ${}_{254}\text{GNLLCQCIC(isoD)/(N)GR}_{265}$  confirming deamidation of the Asn residue (+0.98Da) accompanied by characteristic y ion series (y2–y11, solid red lines), and neutral losses (dashed green lines) of NHCO (43 Da) and NH<sub>3</sub> (17 Da). (C) RAW cells (macrophages) were cultured in 24-well plates coated with either native or deamidated fibronectin for 24h. Exposure to isoDGR-modified protein activated RAW cells via ERK phosphorylation (lower panel) whereas an isoDGR-specific mAb was able to block these effects. Statistical differences between groups were determined by one-way ANOVA. Similar results were obtained using PMA-primed U937 cells (Fig. 3d). (D) U937 cells were reverse-transfected with AP-1 or NF $\kappa$ B-SEAP vector and cultured on native or deamidated fibronectin for 24h. U937 cell adhesion to deamidated fibronectin was associated with marked activation of the AP-1 signal transduction pathway but not NF $\kappa$ B. Each column represents

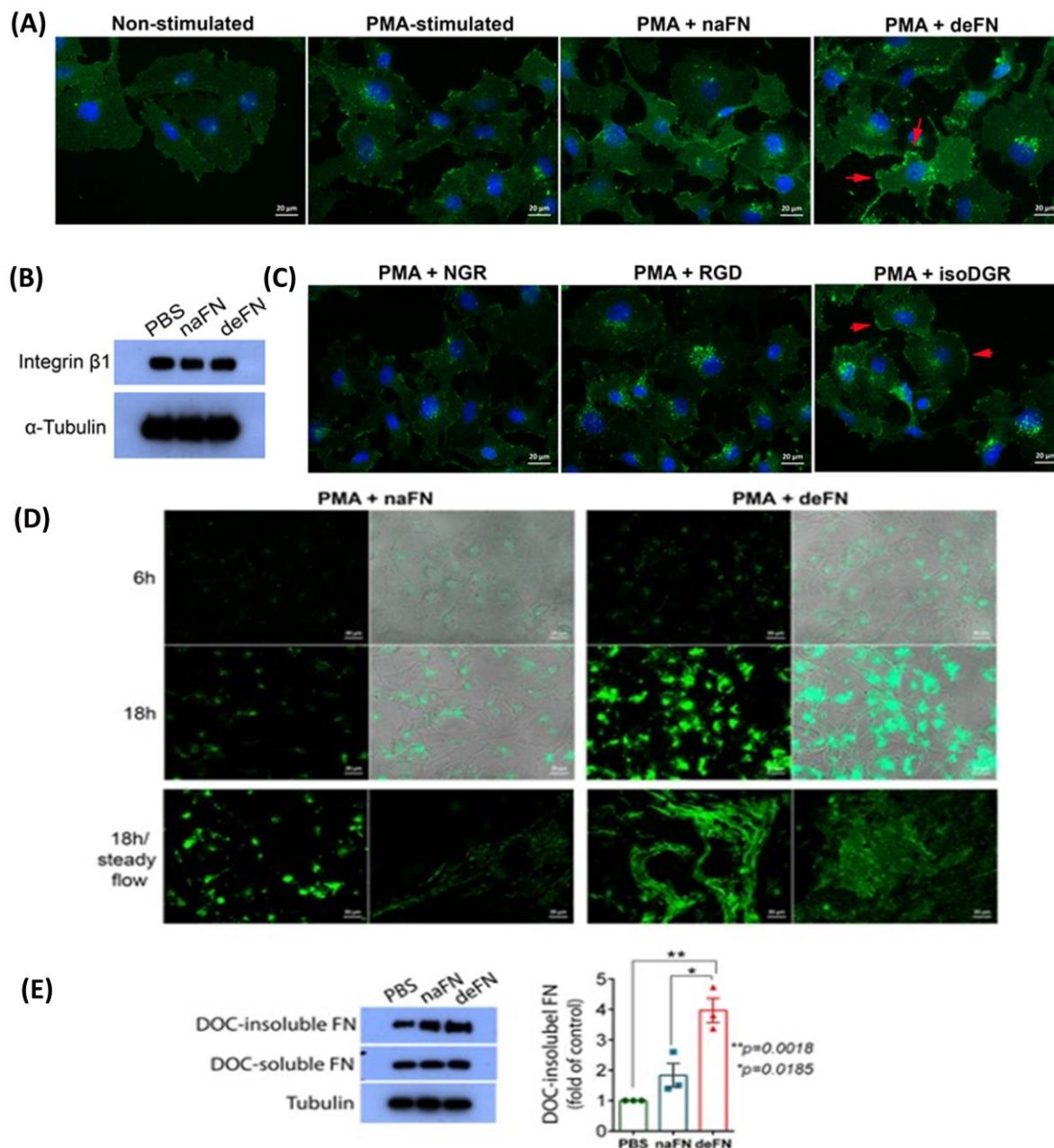


the mean  $\pm$  SE of three biological replicates (two technical repeats per biological replicate). Statistical differences between groups were determined by unpaired T-test.

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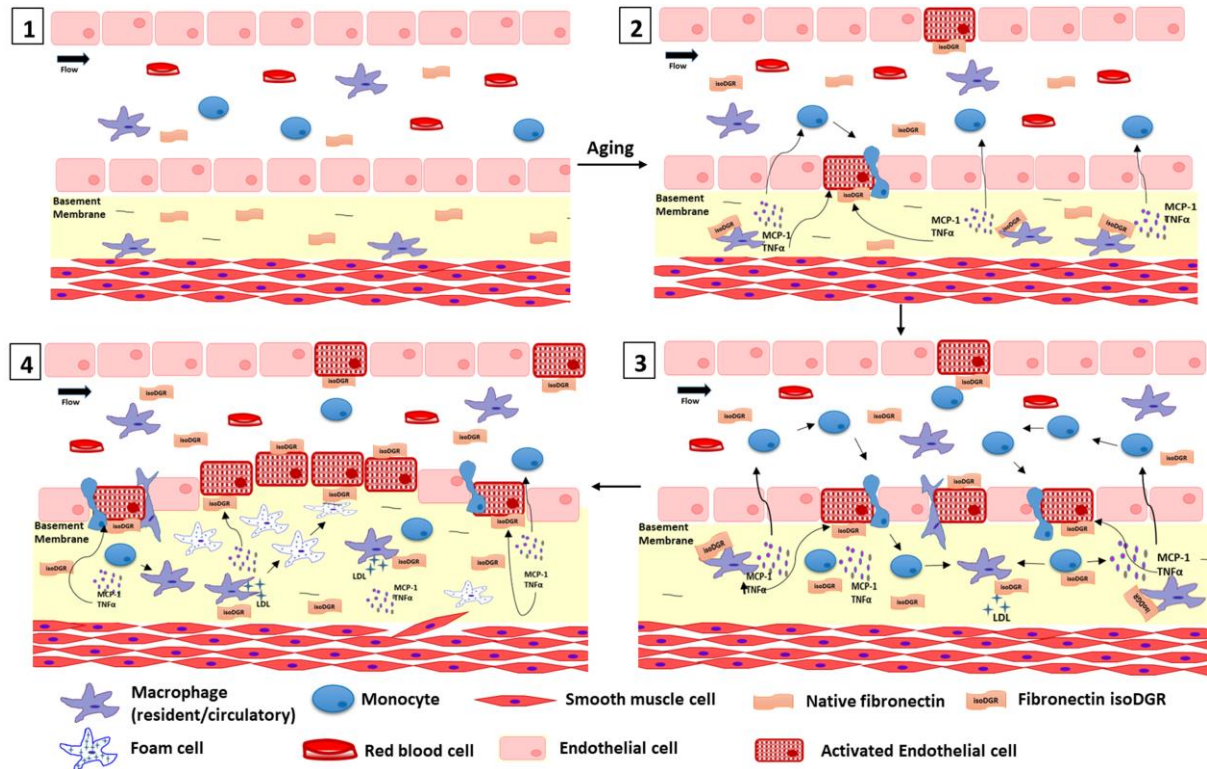
**Figure 3: IsoDGR-modified fibronectin activates macrophages expression of chemotactic MCP-1 and TNF $\alpha$**  (A) RAW macrophage cells were cultured for 24h together with native or deamidated fibronectin before mRNA expression levels of key cytokines were assessed by qRT-PCR. This analysis revealed that macrophages exposed to isoDGR-modified fibronectin displayed significant upregulation of several chemotactic molecules, pro-inflammatory cytokines, and metalloproteinase enzyme MMP9. Experiments included n=3 biological replicates (3 technical repeats per biological replicate, data shown are mean  $\pm$  S.E. and were assessed by unpaired T-test). (B) Legendplex<sup>TM</sup> bead assay of proinflammatory cytokine secretion by RAW macrophages. Exposure to isoDGR-modified fibronectin stimulated macrophage expression of both MCP-1 (>4 fold) and TNF $\alpha$  (>3-fold). (C) In Transwell experiments, adhesion of U937 cells to deamidated fibronectin (bottom chamber) induced greater transmigration of unstimulated monocytes (matrigel-coated top chamber) than did exposure to the native protein control (assessed by microscopic or colorimetric analysis after 24h incubation). All pathological processes described in panels a-c were inhibited by addition of 10 $\mu$ g/ml isoDGR-specific mAb. Experiments included n=5 biological replicates. Statistical differences between groups were determined by unpaired T-test. (D) U937 (PMA- and PMA+) monocytes were cultured in 24-well plates coated with either native or deamidated fibronectin for 24h, during which time ERK phosphorylation was induced only in cells exposed to the isoDGR-modified protein (lower panel). Statistical differences between groups (n=3) were determined by unpaired T-test. Expression of integrin  $\beta$ 3 and pERK were strongly increased when U937 cells (PMA-) were exposed to isoDGR-modified fibronectin, indicating active monocyte differentiation into macrophages. (E) Immunofluorescent imaging of aorta sections (I: intima; M: media; and A: adventitia layers) showing isoDGR-motif distribution and co-localisation of CD68+ macrophages in 8-month-old WT and *PCMT1*<sup>+/-</sup> mice.



**Figure 4: IsoDGR-modified fibronectin stimulated endothelial cell expression of integrin  $\beta 1$  and fibrillogenesis** (A) HUVEC endothelial cells treated with deamidated-fibronectin (25 $\mu$ g/ml) displayed higher levels of integrin  $\beta 1$  activation and distribution to the plasma membrane (red arrows) than were observed among control cells exposed to the native protein (n=3, scale bar 20  $\mu$ m). (B) Total expression levels of integrin  $\beta 1$  were comparable between conditions upon assessment by western blot (n=3). (C) Activation of integrin  $\beta 1$  in these assays required the presence of peptides containing isoDGR motifs but not the alternative sequences NGR or RGD. Key: Native fibronectin, naFN; deamidated fibronectin, deFN (n=3, scale bar 20  $\mu$ m). (D) PMA-primed HUVECs were incubated for 6h or 18h in the presence/absence of either native or deamidated fibronectin (FN) that had been pre-stained with AlexaFluor 488 dye. Accumulation of exogenous fibronectin on endothelial cell surfaces was markedly increased by deamidation (n=3, scale bar 20 $\mu$ m). When the same experiment was repeated under conditions of steady flow (100 rpm for 18h duration), deamidated fibronectin was observed to extend and form stretched fibrils associated with matrix assembly, whereas native protein displayed only limited accumulation on HUVEC monolayers (n=3, scale bar 20 $\mu$ m). (E) Upon completion of the steady-flow assay, HUVECs were treated with 1% DOC to assess matrix solubility, which confirmed that isoDGR-modified fibrils were highly resistant to detergent lysis (n=3 biological replicates).



Quantification of western blot images was by Image J software. Statistical differences between groups were determined by unpaired T-test; \* $p=0.015$ , \*\* $p=0.0018$ .



### Figure 5: IsoDGR-modified fibronectin is a critical mediator of atherosclerotic CVD

Capacity to repair deamidated proteins declines during natural aging, leading to progressive accumulation of isoDGR-modified fibronectin in blood plasma and at the endothelial basement membrane. This age-damaged fibronectin activates tissue-resident macrophages and promotes expression of pro-inflammatory cytokines, particularly chemoattractants MCP-1 and TNF $\alpha$  which promote endothelial activation, vascular inflammation, and further monocyte recruitment. In addition, isoDGR-modified fibronectin can itself directly activate endothelial cells to enhance binding of circulating monocytes via upregulation of integrin  $\beta$ 1. Protein ‘aging’ therefore triggers the early events of endothelial activation and leukocyte recruitment that are required for age-linked pathogenesis of atherosclerotic CVD.

**Point-by-point response to editor / reviewers' comments;**

Reviewers' comments:

Reviewer #1:

This is an interesting paper of large possible interest. However, it is flawed by deficiencies in the presentation, especially in the description of how the experiments were done.

There were no Supplementary Tables 1 and 2 in the materials available to me.

Deamidation of asparagine residues leads to the production of L- and D-aspartate and L- and D-isoaspartate. The authors appear to assume that only L-isoaspartate is formed. This point needs to be addressed in the paper.

Thank you for your positive and insightful comments. We have now added text discussing the production of L- and D-aspartate as well as L- and D-isoaspartate in spontaneous deamidation reactions. However, both non-chiral RPLC-MS/MS and ERLIC-MS/MS methods were not able to separate the L- and D-stereoisomers. Due to the 4000 word limit of research article, the detailed experimental method are submitted as supplementary methods.

**Reviewer's query:**

Methods page 5. The authors write: "The following peptides were produced by GL Biochem Ltd. (Shanghai, China); GC(isoD)GRCGG-(CH<sub>2</sub>-CH<sub>2</sub>-NH<sub>2</sub>), GCGRCGG-(CH<sub>2</sub>-CH<sub>2</sub>-NH<sub>2</sub>), GC(N)GRCGG-(CH<sub>2</sub>-CH<sub>2</sub>-NH<sub>2</sub>), and GCRGDCGG-(CH<sub>2</sub>-CH<sub>2</sub>-NH<sub>2</sub>)."

Figure 1, panel a, where they apparently used these antibodies, there is no mention of the second of these peptides (GCGRCGG-(CH<sub>2</sub>-CH<sub>2</sub>-NH<sub>2</sub>) and there is apparently used there another peptide labeled in the figure "DGR" which I assume has an aspartyl residue and corresponds to the isoaspartyl containing peptide?

Thank you for your careful review. Apologies for this typo error - the original sequence of the peptide is GCDGRCGG-(CH<sub>2</sub>-CH<sub>2</sub>-NH<sub>2</sub>) and has been corrected in the revised manuscript.

**Reviewer's query:**

Figure 1 panel a and legend. The determination of the specificity of the antibody used to probe the isoaspartyl form of fibronectin is crucial to this paper. There are not enough details here or in the methods section to evaluate this specificity. The authors need to clear up the confusion on the synthetic peptides used in the methods section on page 5 and clearly describe what was done in this experiment - including the concentrations of reagents and how the signal was detected.

Thank you for the comments. We have included additional experimental details in the supplementary method section due to length restrictions limit the amount of experimental detail we can provide. Briefly, we used a combination of peptide-based ELISA and dot blot assays to test reactivity of the selected hybridoma cells against four peptides containing either isoDGR or the dummy sequences DGR, RGD, and NGR. IsoDGR-specific antibodies in hybridoma culture supernatants were determined by indirect ELISA.

Briefly, Nunc Maxisorp 96 well plates were coated overnight at 4°C with 100 µL/well of each of the peptides (2 µg/mL). The plates were washed three times in PBS–Tween-20 (0.05%, PBS-T), and then blocked with 5% BSA at 37°C for 2h. The plates were washed three times with PBS-T and 100 µL/well of culture supernatants (1:500) were added and incubated at 37°C for 2 hr. The plates were then washed three times with PBS-T and incubated with 1:5000 diluted goat anti-mouse antibodies (R&D systems, Minneapolis, USA) labelled with horseradish peroxidase (HRP) for 1h at 37°C. After a final three washes with PBS-T, the wells were incubated with TMB substrate (Sigma, USA) and the reaction was terminated by adding 2M sulphuric acid. Absorbance was read at 450nm. Based on the results of these assays, we selected a single hybridoma that displayed high specificity for the isoDGR motif but lacked reactivity to DGR, RGD, or NGR (Fig. 1a). Monoclonal antibody was purified from the supernatant of hybridoma cell cultures using protein A/G agarose columns according to the manufacturer's instructions (Thermo Fisher Scientific, San Jose, USA).

**Reviewer's query:**

Figure 1 panel b. Misspelling of "Indirectt"

Author's response:

Thank you for your careful review. We have corrected the typo and moved the ELISA illustration to supplementary figure 1 as suggested by the second reviewer.

**Reviewer's query:**

Figure 1, panel c. Details are needed of how these measurements were made.

Thank you for your suggestion. We have now repeated and performed additional ELISA experiments, as well as re-run the data analysis and additional statistical data analysis according to clinical and life style features of patients. Intriguingly, smoking status was found to associate with increased levels of isoDGR-modified proteins in CAD patient plasma (see SFigure 2).

**Reviewer's query:**

Figure 1, panels e and f. Details are needed of how these measurements were made.

We have now provided additional details of these ELISA experiments in the supplementary method section.

**Reviewer's query:**

Figure 2. In vitro incubation of fibronectin would be expected to generate many products. The authors need to show evidence for how many products are generated, both by chromatography under conditions that would separate different degraded forms, and then by peptide analysis of each form with the amounts of each peptide indicated by HPLC.

Thank you for your suggestion. We have performed additional LC-MS/MS experiments using both RPLC-MS/MS and ERLIC-MS/MS methods. The abundance of isoDGR-modified FN peptides agreed with our previous publication in Scientific Reports. All the identified FN peptides in native- and deamidated- FN samples, their chromatograms, extracted ion chromatograms (XIC) were submitted with the manuscript in

Supplementary Data 2 to 6. The methods, results, and biological implication have been described in detail in the methods, results, and discussion sections.

**Reviewer's query:**

Comments on the supplemental materials

1. Full unedited gels for Figure 2D, 3C, and 4A. It would be important to also show shorter exposures to insure that the signals are within the (mostly) linear range of the film. Many of the exposures presented here appear to give saturated signals that may not be proportional to the amount of the protein present.

Thank you for these comments. Most of the experiments have now been repeated with additional controls using isoDGR-neutralizing mAb. Please refer to the updated figures and supplementary section of the revised manuscript. All gel images were submitted with the manuscript as SFigure 5.

**Reviewer #2:**

In their study, the authors Park et al. observe increased isoDGR motifs within fibronectin and fibrinogen proteins circulating in plasma samples of 25 patients undergoing coronary artery bypass graft surgery compared to 25 age-matched control subjects, in whom CAD had been excluded by computed tomographic angiography. To examine the potential relevance of their findings for atherosclerosis, they examine human endothelial or monocytic cell lines in vitro and show that exposure to deaminated isoDGR fibronectin induces the exposure of beta 1 integrins on the surface of endothelial cells and exogenous fibronectin binding and fibrillogenesis, whereas monocytic cells react with increased beta 3 expression, AP1 activation and inflammatory cytokine production. They conclude that „Age damaged fibronectin with isoDGR motif increases binding to integrins on the surface of monocytes. Subsequent activation of integrin 'outside-in' signalling pathways elicits a range of potent cytokines and chemokines that drive additional leukocyte recruitment to the developing atherosclerotic matrix and likely constitutes a key early event in progression to CVD."

Overall, the study presents some interesting preliminary data. However, in most experiments positive or negative controls are missing or proof-of-concept analyses are not performed, e.g. using neutralizing antibodies or signal transduction inhibitors in Figure 3A and 3B, Figure 4 and Figure 5. Also, the link between findings in patients with advanced disease and those in monocytic or venous endothelial cell lines using functional assays of early events (adhesion, transmigration) is interesting, but weak and should be further substantiated (e.g. by the analysis of patients' cells, co-incubation studies).

Thank you for your insightful comments and suggestions. We have now repeated most of the functional experiments including isoDGR-specific neutralizing mAb as an additional control. The results clearly show that the observed biological functions/pathological processes observed in macrophage, monocytes and endothelial cells were mediated by isoDGR-modified FN. The manuscript has been substantially revised based on these new results.



**Reviewer's query:**

Main comments:

1. Differences in isoDGR motifs containing plasma fibronectin and fibrinogen levels are reported, but what about total fibronectin and fibrinogen levels in both groups.

Thank you for this insightful comment. We have now repeated the ELISA experiments and included measurement of total plasma FN and FBG proteins as shown in Figure 1 e and f. We did not observe changes in total FN and FBG levels in either group of patients.

**Reviewer's query:**

2. Moreover, and as can be appreciated by the clinical data shown in Table 1, the controls are not healthy, but also display cardiovascular risk factors which should be taken into account when discussing the measurement of isoDGR motifs containing fibronectin and fibrinogen plasma levels for early diagnosis of atherosclerosis (page 15, lines 29-30). Were levels also determined in young healthy persons? This would be interesting to know.

The cardiovascular risk factors evident in the control group are now discussed in the relevant sections of text. In future studies, we do indeed plan to collect samples from young healthy subjects to study isoDGR levels and assess impact on health and disease progression. We have therefore discussed the current limitations of our study in the discussion section. These sentences were added "However, further assessment of this possibility in larger cohorts and prospective studies will now be required since the control subjects used in the current study were limited to individuals with suspected CAD (despite subsequent diagnosis as non-CAD cases by angiography or CCTA)"

**Reviewer's query:**

3. Different terms are used to describe the patients examined in the present study, such as „patients with cardiovascular disease", „CVD patients" and „IHD patients", but also for the control group („non-IHD", „control plasma donors", „control donors" etc.). Only one specific term should be used for each group to avoid confusion. Moreover, and since mechanisms of atherosclerosis are to be investigated (and not the response of cardiomyocytes to ischemia), my suggestion would be to change the term to „patients with coronary atherosclerosis", as this was determined using computed tomographic angiography (CCTA) (page 5, lines 2-3).

Thank you for your very insightful suggestion. We have now amended the text to ensure consistent use of cardiovascular disease (CVD), atherosclerotic CVD, and coronary artery disease (CAD) when describing patients' samples.

**Reviewer's query:**

4. Please also indicate the level of significance in Table 1, even if negative. There appear to be differences in the number of individuals exposed to factors that may affect monocytes recruitment and endothelial cell activation, such as hypertension (76 vs. 20%), smoking (36 vs. 4%), hyperlipidemia (100

vs. 32%) or statin therapy (100 vs. 40%). Were the patients age-matched on a single patient-to-control basis or based on the mean in each group? Please clarify other criteria used for matching test and control persons and also specify exclusion criteria.

Thank you for your insightful suggestion. We agree that the clinical/lifestyle features of the CAD vs non-CAD patients were not well-matched due to limitations in patient recruitment. We therefore performed additional statistical analysis to assess whether clinical and/or lifestyle features affect isoDGR levels. Intriguingly, these analyses revealed that only smoking status was significantly associated with increased isoDGR levels in CAD plasma. These new results are now included in supplementary figure 2.

**Reviewer's query:**

5. Since only 25 patients / controls are examined, the Discussion should be toned down with regard to the usefulness of isoDGR motifs as biomarker or even as marker for early diagnosis or risk prediction.

Author's response:

We have addressed this concern in our answer to question 2. We have also modified the discussion to make more conservative claims regarding the potential utility of isoDGR-based biomarkers in the clinic.

**Reviewer's query:**

6. There is great confusion about the term „vascular fibrosis“, which the authors themselves define as „excess deposition of extracellular matrix (ECM) proteins such as collagen, proteoglycan and fibronectin in the arterial wall“ (page 3, lines 28-30), and their in vitro observation of increased isoDGR containing fibronectin induced monocyte and endothelial cell activation. While fibrosis of the vascular wall including the adventitia is rather a late sequelae of inflammation and observed in advanced atherosclerotic plaques, the observation of fibronectin binding in the present study may be separate from this and probably represents an early event initiating vascular inflammation. Instead of artefically linking fibronectin to fibrosis, the observation rather suggest endothelial and monocyte activation. The title of the manuscript and the schematic drawings (graphical abstract and Figure 6) should be modified accordingly.

Thank you for these very useful comments. We have now removed the fibrosis explanation, and instead focused on how isoDGR-modified fibronectin induces monocyte and endothelial cell activation. Our results do indeed indicate that fibronectin binding likely represents an early event initiating vascular inflammation.

**Reviewer's query:**

7. Moreover, findings of increased fibronectin deposition and fibrillogenesis on endothelial cells and inflammatory cytokine production in monocytic cells should be connected to support the hypothesis of increased inflammatory cell recruitment on FN-activated HUVECs. For example, co-culture studies or ivm in the presence of beta 1 or alpha 5 beta 1 neutralizing antibodies.

We performed qPCR analysis and multiplex cytokine assays to study the increased inflammatory response and cell recruitment using RAW macrophages in the presence/absence of isoDGR-neutralizing antibody.

Multiple cytokines including MCP-1, TNF- $\alpha$ , GM-CSF, CCL4, INF- $\beta$  and metalloprotease 9 mRNA were upregulated several fold in isoDGR-modified FN compared with native FN. Treatment with neutralizing isoDGR-specific mAb controls resulted in reduced mRNA levels of these same mediators. Indeed, as predicted by the reviewer, TNF $\alpha$  and direct interaction of isoDGR-modified FN with endothelial cells activated surface expression of integrin  $\beta$ 1 (known to enhance adhesion via formation of integrin pairs  $\alpha$ 1 $\beta$ 1,  $\alpha$ 2 $\beta$ 1,  $\alpha$ 3 $\beta$ 1,  $\alpha$ 5 $\beta$ 1 and  $\alpha$ 6 $\beta$ 1).

8. As mentioned above, the role of fibronectin should be demonstrated by inhibiting alpha 5 beta 1 integrins directly. What about the role of other fibronectin receptors, such as integrin alpha v beta 3, or other integrin beta 1 ligands, such as collagen, thrombospondin, laminin or vitronectin? Their possible involvement should be at least discussed.

We have now included the following sentences in the discussion section.

Intriguingly, many ECM proteins with native 'RGD' integrin binding motif have 'NGR' sequence motifs susceptible to formation of isoDGR motifs during aging, and were detected in atherosclerotic plaque in our previous study<sup>4-7</sup>. Further investigation of these isoDGR-modified protein function and therapeutic targeting in both animal models and patient tissues should therefore be a priority for future studies.

9. Analyses in vitro were performed in HUVECs, a venous endothelial cell line, to examine pathomechanisms in the arterial system or affecting microvascular endothelial cells. Ideally, key findings should be repeated in arterial or microvascular endothelial cells. At the very minimum, this limitation should be discussed. To the same end, monocyte activation was examined using THP1 and U937 cell lines. What about patient and control monocytes and changes in their activation pattern?

We have now included that following sentences in the discussion section.

Further investigation of these isoDGR-modified protein function and therapeutic targeting in both animal models and patient tissues should therefore be a priority for future studies.

10. Western blot, Figure 4A: The results of the quantitative analysis showing significantly increased integrin beta 3 and phospho-ERK1/2 expression on deFN stimulated HUVEC cannot be recapitulated on the representative Western blot membrane. The bands are rather overexposed (which means that linearity of signals is lost), the data points (n=3 experiments) highly variable. Statistical analysis should be performed using One-Way ANOVA.

All the western blots in figure 4 (figure 2 in revised version) have now been repeated using RAW cells (macrophage cell line) with appropriate controls. The results are the same as the macrophages differentiated from PMA-stimulated U937 cells. Statistical analysis was repeated using One-Way ANOVA. Please refer to results section and figure 2 of the revised manuscript.

11. The abstract needs to be completely rewritten and clearly state the aim, the methods and results of this study, in endothelial cells (currently missing) as well as monocytes. For example, it now says „activation of integrin  $\beta$ 1 in monocytes" although Figure 4 shows integrin beta 3 protein levels. It needs clearly explain which analyses were performed in patients/controls and which in cell lines. In vitro findings of increased THP1 monocyte transmigration can also not be translated in „promoted further leukocyte

recruitment to the assembling plaque." The conclusion („Abstract" and „Highlights") should be written more carefully, as observations in patients and cell lines are still preliminary and do not justify the conclusion that „Damaged fibronectin with isoDGR promotes atherosclerosis through vascular fibrosis" or „thus implicating the deamidated variants in the pathological progression of CVD" (page 11, lines 50-52). For this, time course experiments in animal models of atherosclerosis would have been necessary.

The abstract has been rewritten and updated according to the reviewer's insightful comments and new results included in the revised manuscript. We have observed co-localization of isoDGR-motifs with CD68 positive macrophages in aorta of WT and deamidation-prone murine model.

12. Statistical analysis: Also, ANOVA or appropriate test should be used to compare more than two groups.

We agreed with reviewer's suggestion and have used ANOVA in the statistical analyses. Please refer to the materials and methods section of the revised manuscript.

**Reviewer's query:**

Minor points:

\* The non-original data shown in Figure 1B or 1D could be explained in the text and moved to the Supplement.

They have been moved to the supplementary section.

**Reviewer's query:**

\* Figure 1E and 1F: please use the same y scale to allow the direct comparison of changes.

Author's response:

We have replotted the data with same y-axis scale.

**Reviewer's query:**

\* Why was integrin beta 1 examined on EC and beta 3 on monocytes? Also, why was only fibronectin, what about fibrinogen and platelets?

Accordingly to other previous reports, endothelial integrin  $\beta 1$  enhances adhesion to leukocytes via  $\alpha 1\beta 1$ ,  $\alpha 2\beta 1$ ,  $\alpha 3\beta 1$ ,  $\alpha 5\beta 1$  and  $\alpha 6\beta 1$  receptors on the cell surface. Therefore, we focused only on endothelial integrin  $\beta 1$  expression. We agree with the reviewer, more investigation into isoDGR biology in CVD should be performed in follow-up studies, since the multiple roles of this motif are still not fully understood.

\* Line 7-9: Please change the outdated term „chronic coronary artery disease" to „chronic coronary syndrome" (in line with the ESC guideline 2019).

Author's response:

Thank you for the suggestion. We now consistently use CVD and CAD throughout the manuscript.

Editorial Office comments:

-Atherosclerosis applies formatting guidelines to all accepted papers, with the aim of improving their readability.

Manuscripts that do not conform to the format guidelines of the Atherosclerosis Journal will be returned to the authors for reformatting.

When revising your manuscript, please follow carefully the recommendations of our Atherosclerosis Style Guide to be downloaded from the following link ([http://cdn.elsevier.com/promis\\_misc/Atherosclerosis\\_style\\_guide\\_checklist.docx](http://cdn.elsevier.com/promis_misc/Atherosclerosis_style_guide_checklist.docx)).

- A maximum of 5 figures/tables is required. Move extra data to supplementary files.

- Make sure to apply the formatting requirements to all figures and tables where necessary (e.g. style of p values, gene and protein nomenclature).

- Make sure to use uniform lettering and sizing of your original artwork, including letters to indicate panels, throughout all figures.

- Make sure to submit high resolution versions of each figure.

- A graphical abstract is required at revision.

We have followed the guideline and checklist to format the manuscript, prepare the figures and table. However, due to novelty of the topic and complexity of the experiments, we are not able to write the manuscript within 4000 word limit despite substantial parts of methods, figures and results have been submitted as supplementary materials.

### **Submission declaration**

The authors declare that the work described in this manuscript has not been published previously, except that it was published in the pre-print server bioRxiv:

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The article is not under consideration for publication elsewhere. Publication of the article is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out. If the article is accepted, it will not be published elsewhere by the authors, including electronically in the same form, in English or in any other language, without the written consent of the copyright-holder.

The authors declare that the manuscript reports original works. The manuscript does not have plagiarism, or manipulation or enhancement of data. The original whole gel images are submitted together with the manuscript.

Professor Wolfram Ruf  
Associate editor  
*Atherosclerosis*

24-Feb-2021

Manuscript ID: ATH-D-20-01015

New title: **Aging-induced isoDGR-modified fibronectin activates monocytic and endothelial cells to promote atherosclerosis.**

Original title: Increased isoDGR motifs in plasma fibronectin are associated with atherosclerosis through facilitation of vascular fibrosis *Atherosclerosis*

Dear Professor Ruf,

We would like to thank you and the reviewers for their careful review, insightful comments, and useful suggestions that have helped us to significantly improve our manuscript. Most of the experiments have now been repeated with additional sets of controls using an isoDGR-specific neutralizing antibody. The manuscript has been revised accordingly, and many sections have been rewritten / updated with these additional results. The accompanying new data and subsequent analyses have been included in revised manuscript or as supplementary materials.

Due to novelty of the topic and complexity of the experiments, and the detail information requested by the reviewers, we are not able to write the manuscript within 4000 word limit despite substantial parts of methods, figures and results have been submitted as supplementary materials.

Our point-by-point responses to the reviewers are provided below (in [blue](#)). We have also uploaded the revised manuscript, supplementary materials onto the submission website. We hope this exciting report is now acceptable for publication in *Atherosclerosis*.

Thank you for your consideration. We look forward to the publication of the manuscript in *Atherosclerosis*.

Sincerely yours,

Siu Kwan Sze.

We would like to suggest the following experts to review the manuscript.

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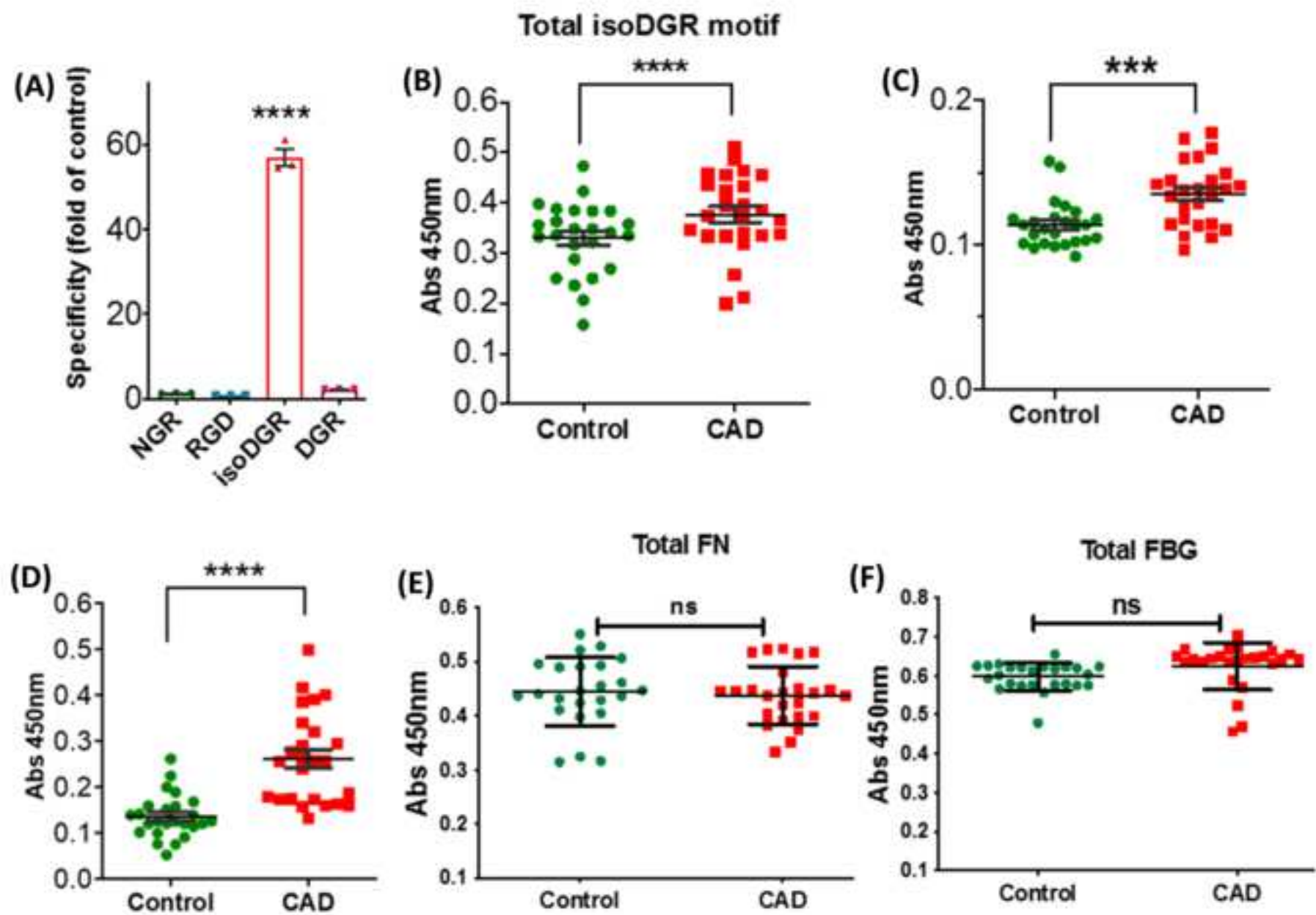


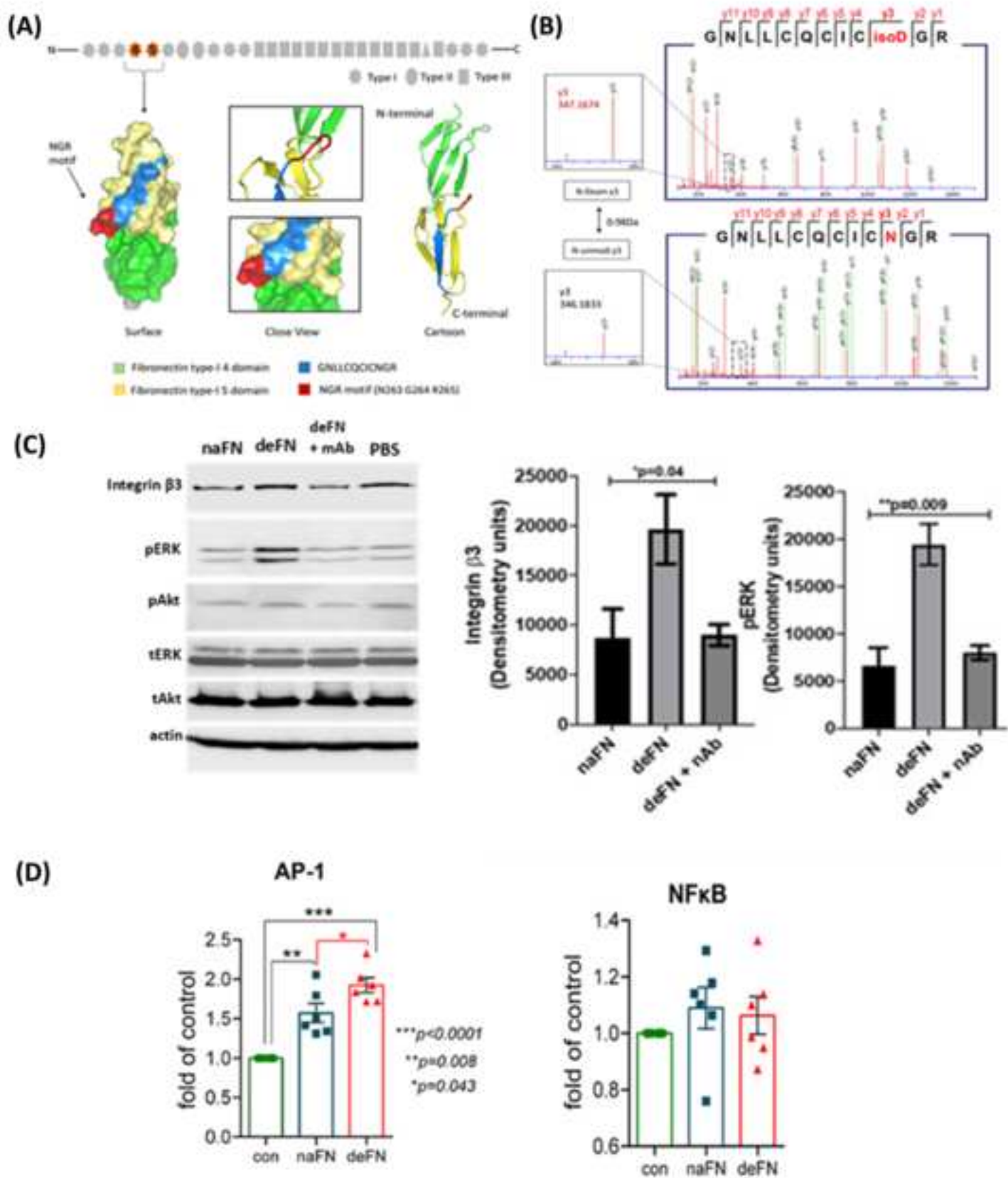
**Increased isoDGR motifs in plasma fibronectin are associated with atherosclerosis through facilitation of vascular fibrosis**

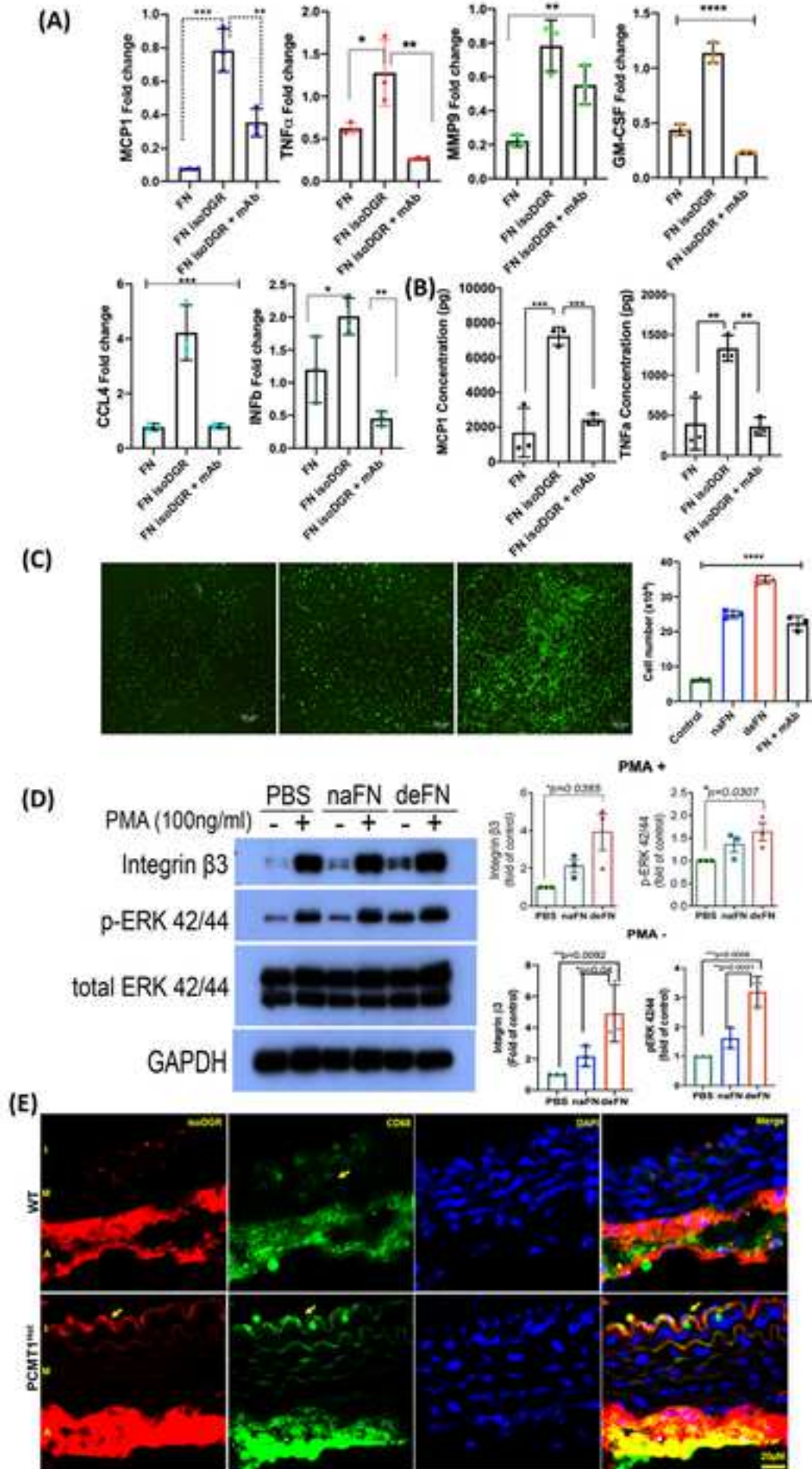
**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

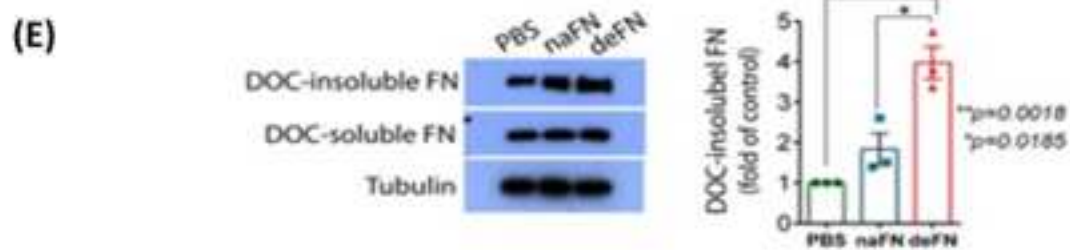
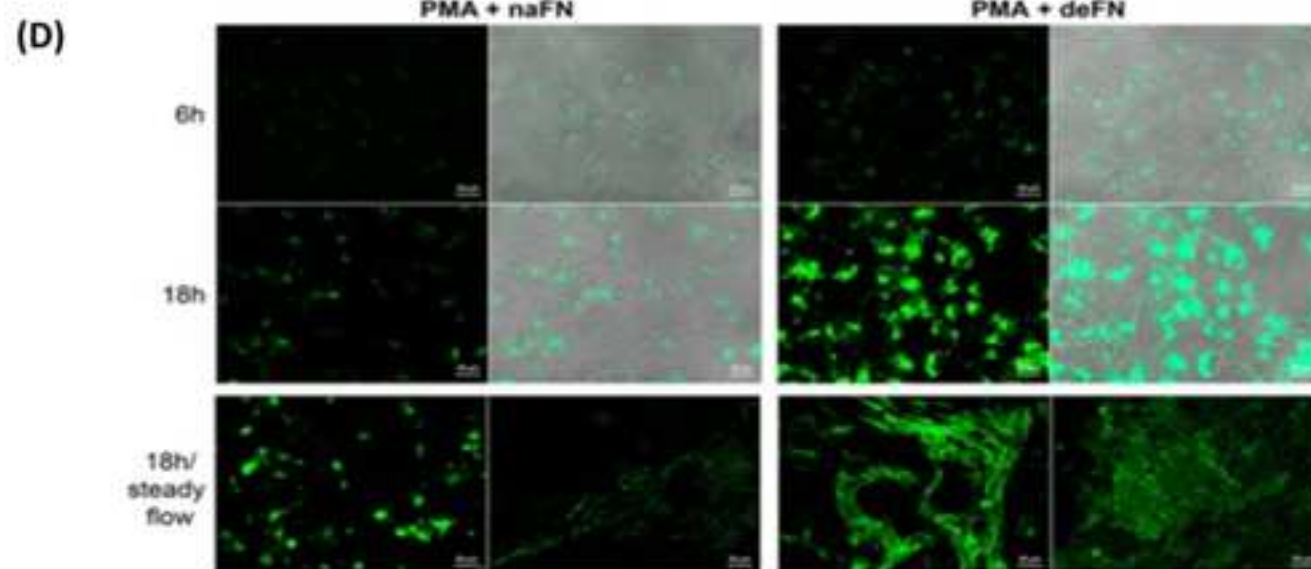
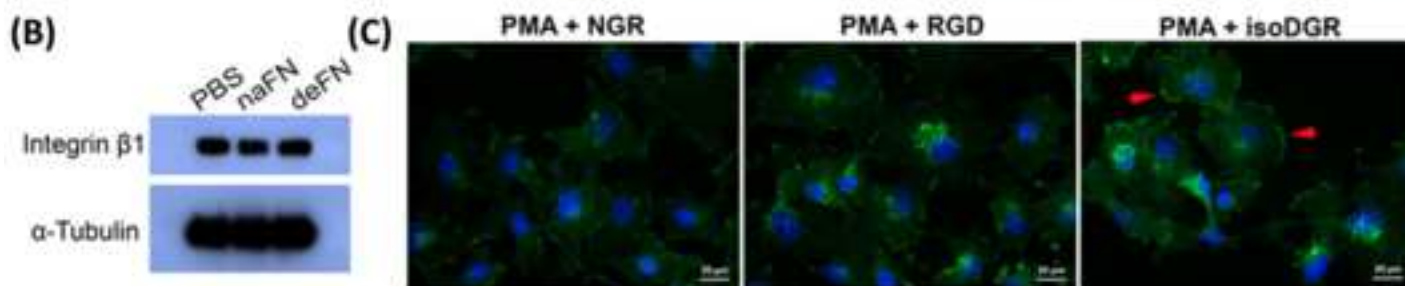
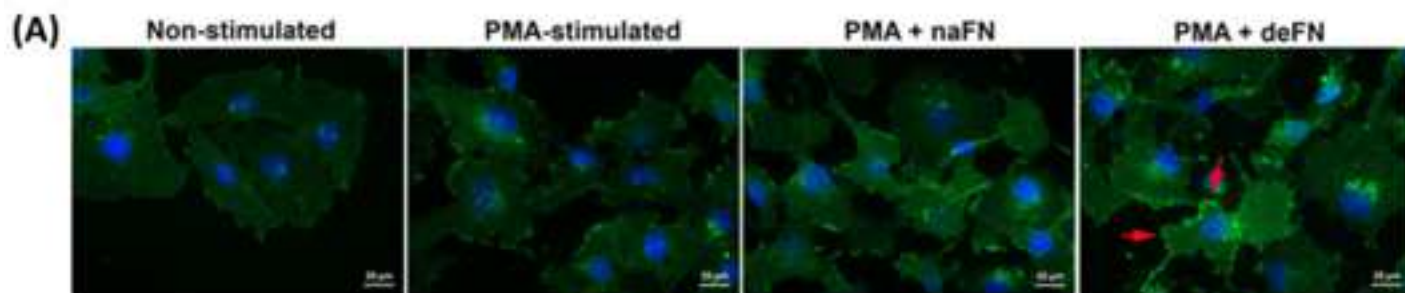
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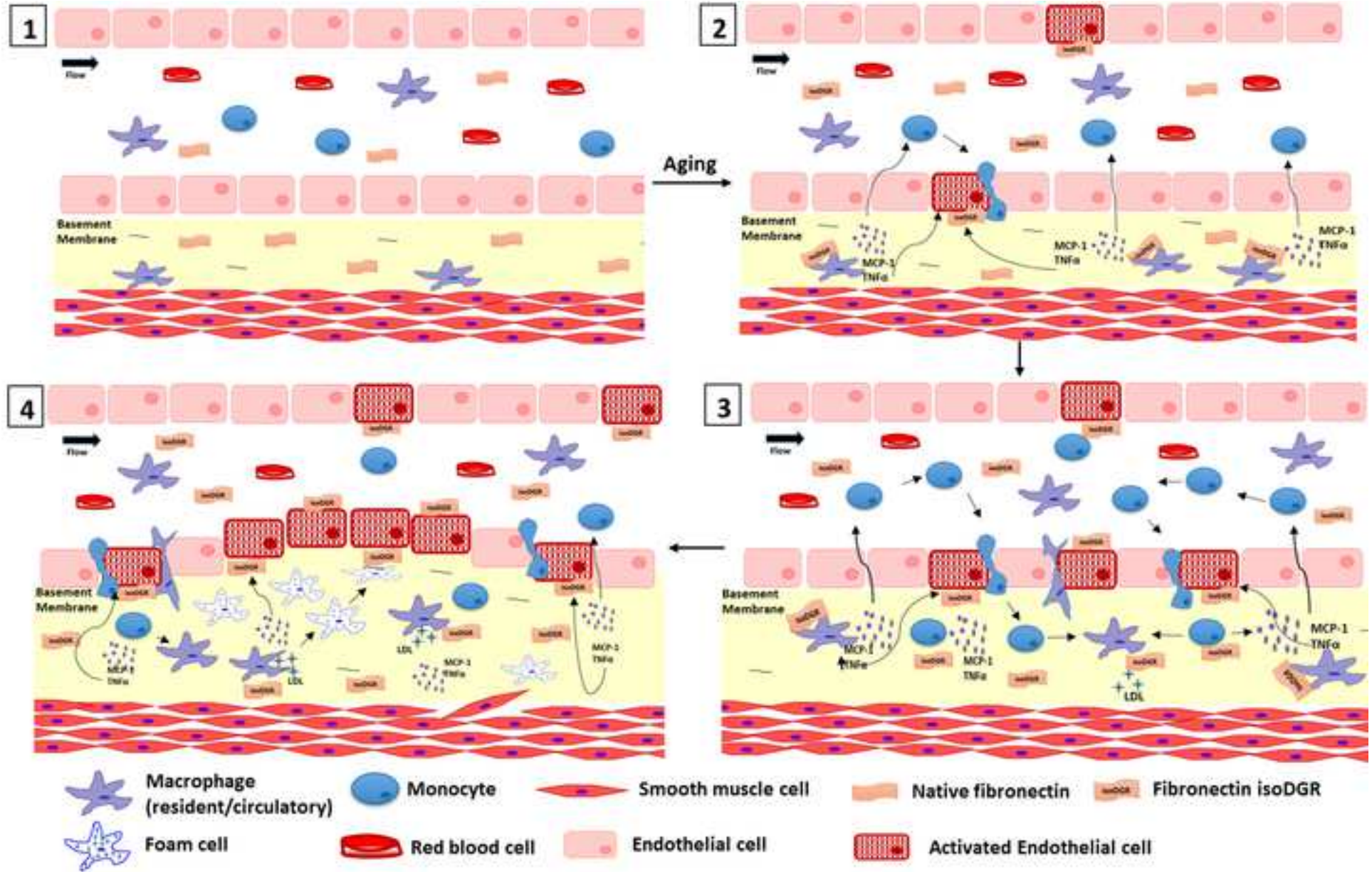


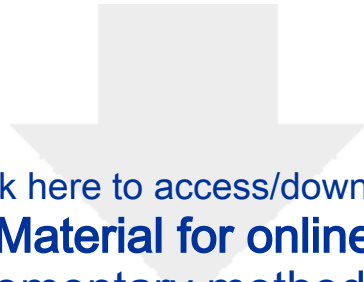












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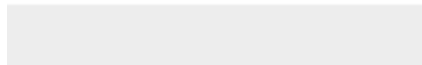
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## **Aging-induced isoDGR-modified fibronectin activates monocytic and endothelial cells to promote atherosclerosis**

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- Abstract in the *Atherosclerosis* format (*Background and aims, Methods, Results, Conclusions*)
- Introduction
- Materials and methods (or Patients and methods)
- Results
- Discussion
- Conflict of interest (mandatory)
- Financial support (if applicable)
- Author contributions (mandatory)
- Acknowledgements (if applicable)
- References
- Figures and Tables (with legends in the suitable style)

#### **Abstract style**

**Is the Abstract structured in the below sections?** **Yes**

- *Background and aims*
- *Methods*
- *Results*
- *Conclusions*

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Each figure and table legend should have a brief overarching title that describes the entire figure without citing specific panels, followed by a description of each panel, and all symbols used.

If a figure or table contains multiple panels, the letter describing each panel should be capitalized and surrounded by parenthesis: i.e. (A)(B)(C)(D).

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#### **Abbreviations**

**Are abbreviations defined when first used in the text?** **Yes**



## **Aging-induced isoDGR-modified fibronectin activates monocytic and endothelial cells to promote atherosclerosis**

Use of abbreviations should be kept at a minimum.

### **Units**

**Are units expressed following the international system of units (SI)?** Yes

If other units are mentioned, please provide conversion factors into SI units.

### **DNA and protein sequences**

**Are gene names italicized?** Yes

Gene names should be italicized; protein products of the loci are not italicized.

For murine models, the gene and protein names are lowercase except for the first letter.  
(e.g., gene: *Abcb4*; protein: Abcb4)

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**Author contributions**

J.E.P. and G.J. performed most *in-vitro* experiments, data analysis and drafted the manuscript; K.P. performed animal experiments and data analysis; X.G. performed LC-MS/MS; S.C.N. performed ERLIC-MS/MS analysis; K.C.K, S.S.N, S.E.L. performed qPCR and WB experiments; W.M. performed MALDI-TOF-MS analysis; S.C.L., M.K.L., D.J.P., D.K., V.S, H.H.H and N.E.M. contributed clinical samples, reagents and revised the manuscript; S.K.S. conceived and supervised the project and wrote the manuscript. All co-authors contributed to the revision of the manuscript.