Blood pressure and TNF-α act synergistically to increase leucocyte CD11b adhesion molecule expression in the BELFAST study: implications for better blood pressure control in ageing

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Abstract Hypertension, a key risk factor for stroke, cardiovascular disease and dementia, is associated with chronic vascular inflammation, and although poorly understood, putative mechanisms include pro-inflammatory responses induced by mechanical stretching, with cytokine release and associated up-regulated expression of adhesion molecules. Because blood pressure increases with age, we measured baseline and tumour necrosis alpha (TNF-α)-stimulated CD11b/CD18 adhesion molecule expression on leucocytes to assess any association between the two. In 38 subjects (mean age 85 years), consecutively enrolled from Belfast Elderly Longitudinal Free-Living Aging Study (BELFAST), baseline and TNF-α-stimulated CD11b/CD18 expression on separated monocytes and neutrophils increased with systolic blood pressure >120 mmHg ($p=0.05$) and for lymphocytes, with diastolic blood pressure >80 mmHg ($p<0.05$). These findings show increased potential stickiness of intra-vascular cells with increasing blood pressure which is accentuated by TNF-α, and suggest mechanistic reasons why better hypertension control is important.

Keywords Blood pressure · TNF-α · Leucocyte adhesion · CD11b/18 · Ageing · BELFAST study

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Introduction

The prevalence of hypertension—a major risk factor for stroke, cardiovascular disease and dementia—increases with age. It reduces healthy life expectancy in ageing populations by contributing in a major way to disability (Hajjar et al. 2007). One of the earliest manifestations of hypertension and associated athero-sclerosis includes endothelial dysfunction in the vascular tree (Krieglstein and Granger 2001). Although the mechanism of its action is not well understood, it has been suggested that hypertension can damage vascular endothelial cells by a combination of shear stress secondary to flow or mechanical stretching related to

increased pulse pressure (Katsumi et al. 2004; Cheng et al. 1996). Changes in adaption induced by disturbed versus laminar flow are therefore key events. Endothelial cells respond to damage, stress or mechanical stretch by up-regulation of cytokines or chemokines mediated through NF-κB, which initiate an inflammatory response in an attempt to delimit damage (Riou et al. 2007; Orr et al. 2005; Dorffel et al. 1999; Albelda et al. 1994).

The integrin family of molecules are αβ hetero-dimers which are important in cell–matrix and cell–cell adhesion functions. They have a common β subgroup (CD18) and one of three possible α subunits (CD11a, CD11b and CD11c). CD11b/CD18 plays an important role in the early recruitment and migration of leucocytes into an inflamed area and is up-regulated on neutrophils and monocytes by ligands such as ICAM-1 on the 'damaged' endothelial cells. The CD11b/CD18/ICAM-1 ligand interaction between leucocytes and endothelium encourages neutrophils to stop, adhere and roll along the endothelium, and monocytes are facilitated to adhere and diapedese between endothelial cells and the extracellular space. Both contribute to the inflammatory response by further up-regulation of chemokine and cytokine mediators within the extracellular space.

Blood pressure increases with age and is present in approximately two thirds of people over the age of 65 (Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure 1997). Furthermore the JNC VII guidelines suggested a category of blood pressure in the 'prehypertension' range associated with increased cardiovascular risk (Chobanian et al. 2003). This has been confirmed by a number of large studies (Lewington et al. 2002; Prospective Studies Collaboration 1995) and most recently by Lawes et al. (2008) who showed excess risk beginning with a systolic blood pressure as low as 115 mmHg, with 54% of stroke and 46% of ischaemic heart disease events occurring in persons with blood pressure in this range.

In this study we measured CD11b/CD18 adhesion molecule expression on leucocytes in very elderly subjects because activated granulocytes and mono-cytes express large amounts of CD11b/CD18 com-pared to the other CD11a/CD18 and CD11c/CD18 integrins. Furthermore, CD11b/CD18 is mainly contained within a large pool of intracellular granules in neutrophils and is up-regulated very manyfold (6 to 8×10^5), by a whole range of inflammatory mediators including cytokines, which is not the case for the other integrins (Mazzone and Ricevuti 1995). We argued that since blood pressure increases with age, there might be evidence of an associated pro-inflammatory response. The cytokine TNF-α, central to the immune response, up-regulates β2 integrin expression in acute and chronic inflammatory clinical situations, and we have investigated its effect on CD11b expression in an 'in vitro' situation, in an attempt to model what may happen clinically.

Here we describe up-regulation of CD11b/CD18 adhesion molecule on leucocytes in older subjects with increasing systolic blood pressure and find that this up-regulation is accentuated by the cytokine TNF-α.

Subjects, methods and materials

Subjects

Subjects were a randomly recruited but consecutive group of 38 elderly subjects, enlisted as part of a longitudinal study of ageing—Belfast Elderly Longi-tudinal Free-Living Aging Study (BELFAST); (Rea et al. 1997, 2009). This study was parallel to but part of the ongoing main BELFAST study. Elderly subjects were recruited with the criteria of the Senieur protocol (Ligthart et al. 1984) and within the time constraint of a master's research project on age-related change in CD11b/CD18 integrin expression (Armstrong et al. 2001). The Senieur protocol, developed in the frame-work EURAGE (Concerted Action Programme on Ageing in the European Community) established exacting admission criteria for human immunogeronto-logical studies, based on clinical and laboratory infor-mation (Ligthart et al. 1984). Subjects enlisted into the BELFAST study were community living, apparently well, independently mobile, mentally competent by Folstein mental assessment (28/30) and able to give written consent (Folstein et al. 1991). Throughout the BELFAST study, general practitioners in Greater Belfast (population approximately 500,000) were invited to enlist nonagenarian, octogenarian and 70-year-old subjects, known to them, who approached 'Senieur' or 'elite' status. Of those willing to volunteer, less than one in four elderly subjects met the criteria. Ethical permission for the study was obtained from the Queens University of Belfast.

Materials and methods

Blood pressure Blood pressure measurements were made in the morning in the subject's home, using a conventional mercury sphygmomanometer and after a 10-min rest, during which the research nurse took a brief medical and drug history from the subject. Classical Korotkoff 1 (systolic) and 5 (diastolic) phases were used to define blood pressure.

Basal expression of CD11b/CD18 on neutrophils, monocytes and lymphocytes Whole blood (10 ml), collected into endotoxin-free tubes containing 25 IU of preservative-free heparin/ml (CP Pharmaceuticals Ltd., Wrexham, UK), was used for flow cytometry analysis of CD11b/CD18 adhesion molecule up-regulation within 2 h of venipuncture. CD11b/CD18 expression on nonstimulated leucocytes was analysed using standard methods—20 μl of phycoerythrin-conjugated monoclonal CD11b antibody (anti-CD11b; Becton Dickinson, San Jose, CA) were added to 100 μl of whole blood. Appropriate controls for nonspecific binding were prepared for each subject. Labelled samples were analysed using FACScan flow cytometry (Becton Dickinson), calibrated using Calibrite beads and the Lysys11software program for acquisition and analysis. Neutrophil and lymphocyte cell populations were identified by combining light-scattering properties with appropriate fluorescent marking (FITC-conjugated monoclonal anti-CD14). A minimum of 10^4 cells was analysed per sample and the cellular fluorescence quantified as median channel fluorescence (MCF).

In vitro stimulation of leucocytes using TNF-α Whole blood incubated for 1 h at 37°C with human recombinant TNF-α (National Institute for Biological Standards and Controls, Potters Bar, UK) was diluted with PBS to low (10 IU) and high (100 IU) concentration. Cells were labelled with anti-CD11b and evaluated as described previously (Armstrong et al. 2001). Whole blood, incubated for 1 h at 37°C, without prior addition of TNF-α served as a negative control to stimulation procedures.

Statistical analysis

Subjects were divided into two groups according to systolic and diastolic blood pressure–systolic blood pressure (<120 and >120 mmHg) and diastolic blood pressure (<80 and >80 mmHg). Differences in measured variables and CD11b/CD18 expression were analysed using the nonparametric Mann–Whitney U test (Table 1) for non-normally distributed data with variables expressed as median and 10th and 90th percentiles. Comparisons for the systolic blood pressure categories for age, systolic and diastolic blood pressure and body mass index (BMI) were made with Student's t test with results expressed as mean and standard deviation. Regression analysis was used to assess any association between blood pressure and adhesion molecule expression. p value<0.05 was considered significant.

Results

Subject characteristics

Table 1 shows the characteristics for measured variables for the 20 male and 18 female subjects with mean age 85 years and grouped according to systolic blood pressure <120 and >120 mmHg. There were no significant differences in total cholesterol, LDL or HDL fractions for subjects with systolic blood pressure >120 mmHg compared to those with blood pressure <120 mmHg, although BMI was higher is the >120 mmHg group. No subject was diabetic.

Smoking and medication

Only four subjects were smokers (Table 1) with comparable percentages for each blood pressure category—one subject in the <120 mmHg (12.5%) and three subjects in the >120-mmHg blood pressure groups (10%). Subject use of aspirin and antihypertensive agent/s is also documented in Table 1. The most commonly prescribed antihypertensive medication was a single agent from the diuretic-related antihypertensive class of drugs, with two subjects in the <120 mmHg and five subjects in the >120-mmHg systolic blood pressure groups using this type of medication. One subject in each blood pressure group used a β-blocker. Two subjects in the >120-mmHg blood pressure group used an angiotensin-converting enzyme (ACE) drug—captopril or lisinopril. An antihypertensive drug from the calcium channel blocker group was used as a single agent by three

Table 1 Subject characteristics by systolic blood pressure greater than and less than 120 mmHg

	Systolic blood pressure <120 mmHg	Systolic blood pressure >120 mmHg	p value
Subject number	8	30	
Sex	4 [M], 4 [F]	16 [M], 14 [F]	
Systolic blood pressure	118.8 [3.5]	143.7 [11.1]	<0.001*
Diastolic blood pressure	78.7 [8.3]	89.0 [6.3]	0.003*
Age (years)	87.6 [5.1]	83.2 [6.9]	0.04*
Cholesterol (mg/dl)	188.7 (170, 216.2)	213.5 (139, 246.3)	0.23
LDL cholesterol (mg/dl)	125.1 (96.9, 145.9)	147.5 (89.6, 174.5)	0.48
HDL cholesterol (mg/dl)	31.0 (24.2, 59.8)	40.9 (30.4, 60.2)	0.18
Urea nitrogen (mg/dl)	17.2 (12.4, 23.6)	18.2 (11.6, 31.1)	0.50
Glucose (mg/dl)	79.3 (74.4, 107.4)	82.9 (72.1, 100.2)	0.67
BMI	22.5 [1.64]	25.9 [4.1]	0.02*
Smoking	1 [12.5%]	3 [10%]	
Hypertensive drugs			
Diuretic related	2	5	
β-blockers	1	1	
ACE	0	2	
Calcium CB	0	3	
Combinations	0	3	
Aspirin	0	4	

Mean and standard deviation are indicated in square brackets, p values by t test. Median and 10th and 90th percentiles in parenthesis, p values by Mann–Whitney U. To convert values of urea nitrogen to millimoles per litre multiply by 0.357. To convert values of glucose to millimoles per litre multiply by 0.0555. To convert values of cholesterol, LDL and HDL to millimoles per litre multiply by 0.259

M male, *F* female, *BMI* body mass index, *ACE* ACE inhibitor, *Calcium CB* calcium channel blocker, *Combinations* >1 hypertensive drug

*p<0.05 significant

other subjects in the >120-mmHg blood pressure group. Three subjects in this group used a combination of more than one antihypertensive agent. No subject in the <120-mmHg blood pressure group used aspirin, whereas four subjects in the >120-mmHg blood pressure group used this preparation, usually in a dose of 300 mg, as was more common at the time of recruitment.

Effect of TNF-α 10 and 100 IU

Flow cytometry results are expressed as MCF with box and whisker plots (Fig. 1). Expression of CD11b on the surface of neutrophils, monocytes and lymphocytes increased from baseline in a dose-dependent manner after stimulation with 10 and 100 IU amounts of TNF-α, respectively. Neutrophils and monocytes

demonstrated more CD11b expression (approximately 10-fold) compared to lymphocytes (Fig. 1) both at baseline and with 10 and 100 IU of TNF-α.

Association with blood pressure

Figure 2 shows the regression line and association between increased CD11b expression on neutrophils (R^2=0.12, p=0.04) and monocytes (R^2=0.11, p=0.05) and increasing systolic blood pressure. Subjects, categorised by systolic blood pressure >120 and <120 mmHg, showed significantly higher baseline and 10 IU TNF-α stimulated CD11b expression on both neutrophils and monocytes (p<0.05), though this effect became attenuated at the 100 IU concentration of TNF-α (Fig 1). Similarly, lymphocytes showed significantly higher CD11b baseline up-regulation for
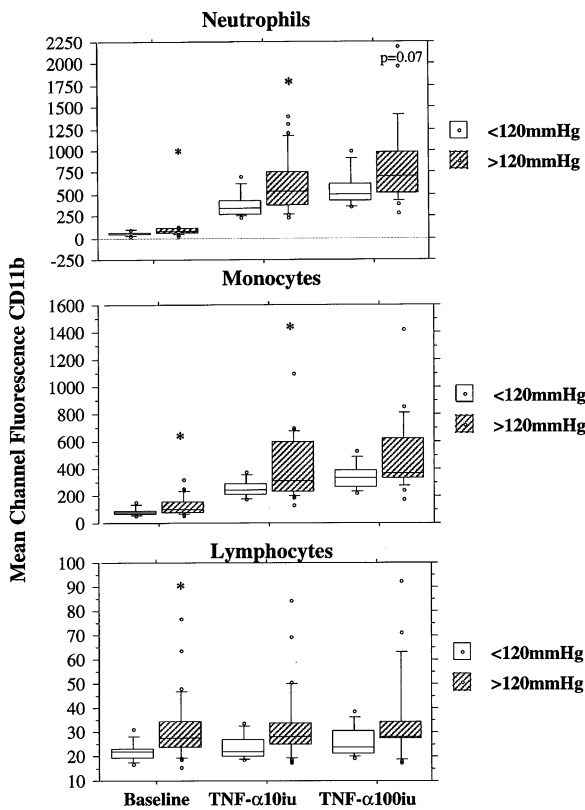
Fig. 1 Mean channel fluorescence CD11b for neutrophils, monocytes and lymphocytes categorised by systolic blood pressure <120 and >120 mmHg showing box and whisker plots for median and 25th and 75th percentiles
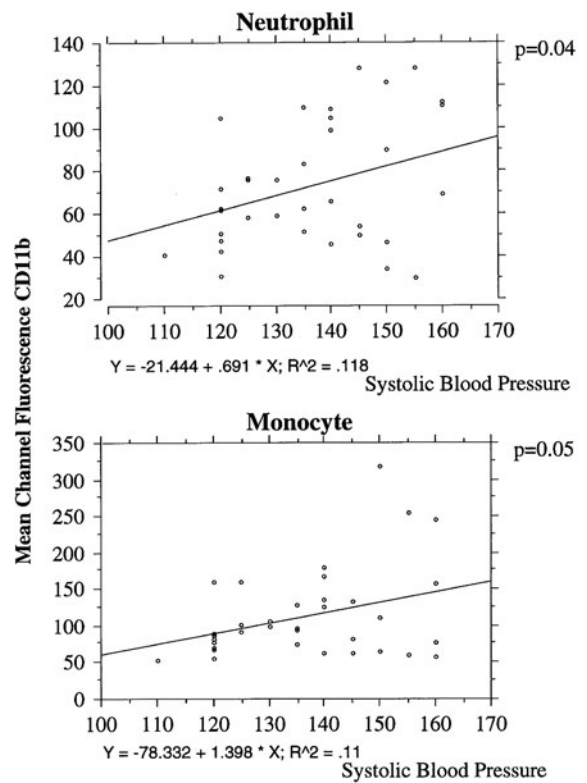

Fig. 2 Regression line and association between increasing systolic blood pressure and mean channel fluorescence CD11b for neutrophils and monocytes with R^2 values

subjects with systolic blood pressure >120 mmHg compared with <120 mmHg, although no increased effect was seen with TNF-α. Diastolic blood pressure >80 mmHg was associated with a modest but significant up-regulation of baseline and TNF-α stimulated CD11b expression on lymphocytes alone (Fig. 3). No changes were seen for any increase in diastolic blood pressure for neutrophils or monocytes.

Discussion

The most important finding in this study in elderly BELFAST subjects was the apparent association between up-regulation of the $\beta2$ integrin adhesion molecule CD11b on neutrophils, monocytes and lymphocytes in subjects who also had systolic blood pressure greater than 120 mmHg. This fits with the putative suggestion that hypertension may induce a pro-inflammatory response in the endothelium

induced by mechanical stretching or pulse pressure changes (Krieglstein and Granger 2001; Katsumi et al. 2004) and is consistent with 'in vitro' work showing that stretching of endothelial cells stimulates cytokine release (Cheng et al. 1996).

There is a large body of accumulating evidence from epidemiological studies and meta-analyses,
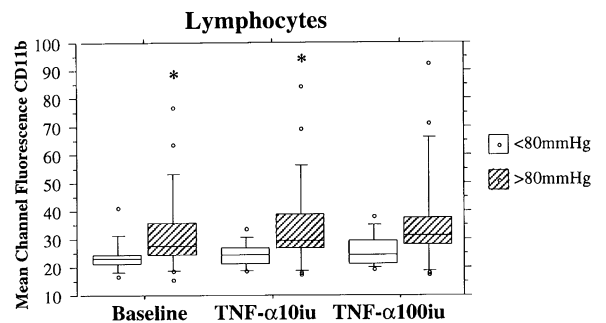

Fig. 3 Mean channel fluorescence CD11b for lymphocytes categorised by diastolic blood pressure <80 and >80 mmHg showing box and whisker plots for median and 25th and 75th percentiles

showing that systolic hypertension has a strong, consistent and graded influence on mortality and morbidity from cardiovascular disease, stroke and end-stage renal disease. For each 20 mmHg increase in systolic blood pressure and 10 mmHg increase in diastolic pressure greater than 115/75 mmHg, there was a twofold increase in mortality associated with stroke and cardiovascular disease (Lewington et al. 2002). Although arguments continue about the blood pressure category 'prehypertension' (Chobanian et al. 2003), subsequent studies, including the recent meta-analysis by Thompson et al. (2011), show clear evidence that increases in blood pressure above 120/80 mmHg (prehypertension range) are associated with continuous, graded and independent increased risk of atherosclerotic-related events (Lewington et al. 2002). Contrary to original opinion, the association of systolic blood pressure with cardiovascular and renal disease is much stronger than the corresponding relationship for diastolic blood pressure. In this context, it is of interest that in the current study, only lymphocytes showed an increase in baseline CD11b expression with diastolic blood pressure above 80 mmHg, whereas all three cell types showed an increased response for systolic blood pressure above 120 mmHg. For the BELFAST subjects in this study of mean age 85 years, increased adhesion molecule up-regulation occurred with blood pressures within and above the range of blood pressure of 120/80 mmHg.

One other related finding is that CD11b up-regulation was accentuated on neutrophils and monocytes by prior 'in vitro' stimulation with TNF-α, a cytokine which stands at the centre of the inflammatory response. TNF-α is produced early in inflammation from a wide range of cells but mainly from monocytes and is up-regulated in both acute illnesses such as chest infection, and a whole range of chronic inflammatory diseases such as rheumatoid arthritis. In the present study, prior stimulation of neutrophils and monocytes with 10 and 100 IU of TNF-α produced large increases from baseline CD11b expression (100-fold for monocytes), which were further increased (approximately by 25%), in subjects with systolic blood pressure >120 mmHg. Others have noted pre-activation of monocytes in subjects with hypertension with accompanying cytokine changes (Dorffel et al. 1999; Dalekos et al. 1997). The current finding seems likely to have important implications for clinical

situations. It suggests that acute or chronic inflammation capable of increasing TNF-α could greatly increase adhesion molecule up-regulation in subjects with even moderately increased systolic blood pressure. The augmented pro-inflammatory milieu could lead to increased vascular risk associated with inflammation. It is already recognised that management of chronic diseases such rheumatoid arthritis which carry an increased vascular risk can be ameliorated by use of various anti-TNF-α direct and indirect monoclonal antibodies (Jacobsson et al. 2005).

This study has a number of limitations. Blood pressure was measured only once using a conventional mercury sphygmomanometer and Korotkoff sounds, with each subject resting for 10 min prior to measurement. With respect to a single blood pressure measurement using the Korotkoff method, evidence from Lewington et al. (2002) of nearly one million people in 61 studies showed remarkably similar blood pressure measurements for seated subjects, when using either a standard or random zero sphygmomanometer, with Korotkoff sounds used to define systolic and diastolic phases. This study further noted that if '[a] single measurement of blood pressure [was] used to predict risk then, irrespective of age, systolic blood pressure was more informative than diastolic pressure'. Staessen et al. (1999) too advised that the Riva Rocci/Korotkoff technique, although prone to error, was easy and cheap to perform and remained worldwide, the standard procedure for measuring blood pressure. Our choice of 10 min rest was both pragmatic—in order not to tire elderly subjects and return samples promptly for processing—and evidence-based, as Sala et al. (2006) demonstrated, on the basis of their repeated measurement of blood pressure at clinic, that 75% of the spontaneous fall occurred within 10 min. They suggested that 10 min rest before blood pressure measurement at clinic could improve precision and accuracy. Our subjects were seen in the familiar environment of their own home, and this fits with evidence showing that home measurement provides better sensitivity for ruling out hypertension, compared to clinic measurement (Hodgkinson et al. 2011).

The numbers of older subjects involved in this study could have reduced the overall statistical power. Recruitment was slow because subjects were elderly with a mean age of 85 years, were community living and relatively few could meet both the 'elite' criteria

of the BELFAST (Rea et al. 1997, 2009) and the exacting demands of the Senieur protocol for immune-gerontological studies (Ligthart et al. 1984). The subject group enrolled from within the ongoing BELFAST study was also subject to time constraints imposed by the completion of a master's research project (Armstrong et al. 2001). Findings, therefore, need to be replicated by other groups. Research studies at this age remain scarce but give valuable insights into factors related to the demographic change evident around us. However they are challenging because of subject frailty, real or perceived vulnerability and lack of family understanding about autonomy of research participants (Samuelson et al. 2008). These factors contributed importantly to recruitment success and time delays.

Hypertension is a silent disease with poor compliance (Knight et al. 2000). The oldest age group has both the highest incidence of hypertension and risk of vascular events including stroke (Hyman and Pavlik 2001; Lewington et al. 2002; Kannel et al. 2008; Clarke et al. 2002; Cooperative Research Group 1991). Understanding how hypertension produces vascular damage and drives cellular adhesion is important since it can help direct doctor and patient treatment strategies and improve compliance. There is increasing evidence that adhesion molecules can be blocked or down-regulated by a range of pharmacological and nonpharmacological mechanisms. The work of Link et al. (2006) found that CD11b expression was inhibited by angiotensin 11 receptor blockers and suggested that this effect was mediated by blockage of the probable paracrine, autocrine and intracine pro-inflammatory vascular mechanisms, driven by the renin–angiotensin system. Additionally, aspirin often used in hypertension because of its anti-platelet effect has been shown to reduce monocyte adhesion by inhibiting the NF-κB pro-inflammatory pathway (Weber et al. 1995; Eisele et al. 2004). There is also recent evidence from animal models that aspirin acts synergistically with angiotensin 11 receptor blockers to protect the vasculature from angiotensin 11-induced organ damage (Muller et al. 2001; Mulay et al. 2010). Nonpharmacological substances such as vitamin C also suppresses TNF-α-induced NF-κB-activated expression by an antioxidant-related mechanism (Carcamo et al. 2002) and resveratrol, from red wine, blunts TNF-α-induced monocyte adhesion (Kim et al. 2007). In this context it is of interest that the Thompson meta-analysis (Thompson et al. 2011), demonstrated in 63,259 participants, who received either an angiotensin-converting enzyme inhibitor, an angiotensin receptor blocker or combination antihypertensive therapy including an angiotensin-converting enzyme drug that there was a significant reduction in risk of stroke, congestive heart failure, cardiovascular events and all cause mortality compared with those not receiving angiotensin-converting enzyme-related antihypertensive therapy, even when blood pressure was not in the hypertensive range. The authors note that their 'meta-analysis was not a mechanistic study and could not therefore determine whether the benefit associated with the use of antihypertensive treatment was attributable to blood pressure lowering or to other tissue or neurohormonal mechanisms'. However it could be argued that reducing cellular adhesion could have been a contributory factor to the improved outcomes. Importantly, in the present study, aspirin and/or ACE inhibitor use was confined to subjects in the >120-mmHg blood pressure group, where they could have potentially attenuated CD11b expression.

This study, in older subjects, suggests an association between increases in blood pressure and increased adhesion on leucocytes. Stickiness was further enhanced by TNF-α prestimulation, a situation likely clinically in association with any acute or chronic inflammation. Populations of octogenarians and nonagenarians are the fastest growing sector of western populations, and their vasculature is at risk. Stroke, the most tragic consequence of untreated hypertension (Kannel et al. 2008), increases with age and robs people of their autonomy. These findings support calls for better control of blood pressure and to a lower level (Beckett et al. 2008; Meissner et al. 1999), irrespective of age. Perhaps then better quality ageing and the 'longevity dividend' can become a reality for everyone (Butler et al. 2008; Bennati et al. 2010).

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