Annexin A5 in treated hypertensive patients and its association with target organ damage

Alessandro Maloberti\textsuperscript{b}, Paolo Meani\textsuperscript{a,b}, Paola Vallerio\textsuperscript{a}, Marisa Varrenti\textsuperscript{a,b}, Francesca Casadei\textsuperscript{a}, Francesco Musca\textsuperscript{a}, Rita Facketti\textsuperscript{b}, Anna M. Di Blasi\textsuperscript{a}, Susanna Ravassa\textsuperscript{d}, Giuseppe Mancia\textsuperscript{b,c}, and Cristina Giannattasio\textsuperscript{a,b}

Objective: Annexin A5 (AnnA5) has been previously linked to the presence of carotid and cardiac target organ damage (TOD) in the context of heart failure and rheumatologic patients. However, information is scant in the context of hypertension. Aim of our study was to evaluate AnnA5 in treated hypertension patients compared with normotensive controls and to determine whether it is associated with vascular and heart TOD evaluated as arterial stiffness, carotid plaque and left ventricular hypertrophy.

Methods: We enrolled 123 consecutive treated hypertension and 124 normotensive controls. TOD was evaluated as pulse wave velocity (PWV, cm/s), left ventricular hypertrophy (echocardiography) and intima–media thickness and carotid plaque presence (ecographic methods). AnnA5 levels was dosed and compared in patients with and without hypertension and with and without TOD.

Results: With similar age hypertension patients showed higher SBP, DBP and AnnA5 levels (13.9 ± 11.1 vs 10.1 ± 8.4 ng/ml, \(P < 0.001\)) compared with controls. Regarding TOD hypertension showed higher PWV (8.5 ± 1.8 vs 7.6 ± 1.5 m/s, \(P < 0.001\)) and LVMI (121.7 ± 29.3 vs 113.5 ± 21.1 g/m^2, \(P < 0.05\)), whereas carotid intima–media thickness was superimposable. AnnA5 correlates with PWV (\(r = 0.13, P < 0.05\)) and DBP (\(r = 0.15, P < 0.01\)), whereas it has never been found as a significant independent predictor of TOD in linear regression analysis.

Conclusion: Our data have shown that AnnA5 levels are increased in treated hypertension patients. In this condition, it is probably released in the plasma as a defensive mechanism through its anti-inflammatory and anticoagulants effects. We found a significant association with arterial stiffness, but AnnA5 was not found to be a significant predictor of TOD.

Keywords: annexin, arterial hypertension, arterial stiffness, carotid plaque, left ventricular mass index, pulse wave velocity, target organ damage

Abbreviations: AnnA5, annexin A5; BP, blood pressure; EF, ejection fraction; HT, hypertension; IMT, intima–media thickness; LVEDD, left ventricular end-diastolic diameter; LVH, left ventricular hypertrophy; LVMI, left ventricular mass index; LVM, left ventricular mass; PWV, pulse wave velocity; TOD, target organ damage

INTRODUCTION

Arterial hypertension is the most important cardiovascular risk factor and the relationship between blood pressure (BP) value and increased morbidity, and mortality has been extensively studied [1]. Pathological heart, vascular and kidney involvement [also known as target organ damage (TOD)] are the linking between the increased afterload determined by hypertension and the clinical events observed via various mechanisms.

Arterial stiffness (i.e., a vascular TOD) is determined by changes in vascular structure and function such as a progressive decrease in elastin/collagen ratio, reduced smooth muscle cell proliferation and impaired endothelial mediated vasodilation, as a consequence of pathological processes mediated by mechanical, neurohumoral and inflammatory routes [2]. Similar mechanisms are implicated in left ventricular hypertrophy (LVH) that is characterized by a complex remodeling of the myocardial structure such as enhanced cardiomyocyte growth, excessive cardiomyocyte apoptosis and accumulation of interstitial and perivascular collagen fibers [3]. Both arterial stiffness and LVH are TOD, and their presence is associated with a demonstrated increase in morbidity and mortality [1,4–8].

Annexin A5 (AnnA5) is a calcium-dependent phospholipid-binding protein, highly expressed by endothelial cells [9]. Under stress conditions, AnnA5 is produced and released into the extracellular medium and bloodstream.
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[10] in which it can exert its anti-inflammatory, anti-apoptotic and anticoagulant properties by shielding the stressed or dying cells from inflammatory cell contact [11,12]. It has been previously linked to the presence of carotid TOD [both as carotid plaques and increased carotid intima–media thickness (IMT)] and cardiac TOS (as LVH) in the context of heart failure and systemic lupus patients [13–16]. However, information is scant in the context of hypertension with only one previous study that found a higher AnxA5 levels in hypertension patients with higher level of this marker on myocardial biopsy in patients with LVH [14].

The aim of our study was to evaluate serum AnxA5 in treated hypertension patients compared with normotensive controls and to determine whether this circulating protein is associated with vascular and heart TOD evaluated as arterial stiffness, carotid plaque and LVH. Evaluation of arterial stiffness relationship with AnxA5 is particularly important as it has never been studied before.

METHODS

Study population

As previously described [17] from September 2006 to October 2008, we enrolled 123 consecutive 18–80 years aged outpatients, followed by the Hypertension Unit of S. Gerardo Hospital (Monza, Italy) affected by essential hypertension. A total of 124 patients, taken from the blood donor list of the hospital, served as control group and were matched for age and sex.

Exclusion criteria for both groups were age less than 18 years, pregnancy, secondary hypertension (investigated by appropriate biochemical and instrumental assessment), chronic kidney and pulmonary disease, substance abuse, history of cancer and patients with cardiovascular events in the month before the study [myocardial infarction (MI), angina pectoris, heart failure, stroke, transient ischemic attacks and claudication].

We collect, in all patients, a complete medical history including drugs use (antihypertensive, lipid-lowering drugs and NSAID) and perform a complete physical examination. All the patients were under antihypertensive treatment (and thus were known as hypertension patients) since at least 2 years. With the patient in a sitting position for at least 5 min and with the arm placed at heart level, four clinic BP measurements were taken by a trained physician: two manual by mercury sphygmomanometer and two with a semi-automated device (OMRON Healthcare Europe, Hoofddorp, The Netherlands). The average of the two kinds of measurements was used for statistics. Hypertension was defined as SBP of at least 140 mmHg and/or DBP of at least 90 mmHg or as the reported use of antihypertensive drugs.

We measured fasting blood glucose, serum total cholesterol (TC), HDL, LDL cholesterol, serum triglycerides and serum creatinine. Estimated glomerular filtration rate (eGFR) by the chronic kidney disease (CKD) epidemiology collaboration (CKD) equation [18] and CKD was defined as a eGFR less than 60 ml/min. Height and weight were obtained to calculate the patient BMI, and waist circumference was assessed halfway between the lower ribs and the iliac crest.

Measurements of pulse wave velocity (PWV), left ventricular mass index (LVMi) and IMT were made by two operators unaware of the patient’s clinical status, two measurements were obtained in each patient, and the mean was used for the analysis.

Annexin A5 measurement

Blood samples were withdrawn from the left antecubital vein and stored at −20°C. Blood was centrifuged and plasma aspirated and stored at −80°C. Plasma AnxA5 was measured by using an AnxA5-specific ELISA (Zymutest Annexin V, Hyphen BioMed, Neuville-sur-Oise, France) as previously described [14]. The interassay and intra-assay variations for determining AnxA5 were 8.4 and 3.5%, respectively. The sensitivity was 0.1 ng/ml.

Pulse wave velocity

Aortic stiffness was evaluated by pulse wave velocity (PWV) between the carotid and the femoral artery of the same side with the patient in the supine position. The pressure pulse waveforms were simultaneously obtained at the two arterial sites at the right side using an automatic device (Compilor, Colson; Alam Medical, Paris, France) and their distance calculated by taking the distance between hip and neck via a rigid ruler. Measure was corrected by a 0.8 factor according to the PWV measurement methods consensus documents which state to use the subtraction methods instead of the direct one when assessing the distance between the two measurements points [19]. In our laboratory, the intra-session within-operator and between-operator variability of PWV amounts, respectively, to a coefficient of variation of the mean value of 2% and to 4%, the corresponding value for the inter-session between-operator variability being 4%.

Arterial stiffness was defined as a PWV measurement higher than 10 m/s according to current guidelines [19].

Left ventricular mass

Two-dimensional echocardiograms were performed by an experienced cardiologist using a dedicated ultrasound machine (SONOS 5500; Philips Healthcare, Andover, Massachusetts, USA with an ultrasound transducer of 2.5 MHz.) in each patient. Two-dimensional high frame rate gray-scale loops of four-chamber, two-chamber and three-chamber views with an average frame rate of 90 frames per second (fps) were used to measure left ventricular end-diastolic diameter (LVEDD), interventricular septum, posterior wall thickness and ejection fraction by the Simpson method. Left ventricular mass (LVM) was calculated using the Devereux formula [20]:

\[
LVM \ (g) = 0.8 \times 1.04 \times \left( \frac{LVEDD \ (cm)}{C_1} \right)^3 \times \left( \frac{C_2}{8} \right) \times \left( \frac{C_3}{8.04} \right)^{-2} \times \left( \frac{C_1}{15} \right) + \ \text{interventricular septum} + \ \text{posterior wall thickness (cm)}^2 \times C_5 + 0.6.
\]
The LVM values were normalized for BSA to obtain left ventricular mass index (LVMI). We calculated BSA using the DuBois and DuBois formula:

\[
BSA \ (m^2) = 0.007184 \times \text{height} \ (cm) \times 0.725 \times \text{weight} \ (kg) \times 0.425.
\]

The intraoperator variability in terms of coefficient of variation of the mean of two measurements is less than 3% in our laboratory.

LVH was diagnosed by LVMI of at least 115 g/m² for men and at least 95 g/m² in women [21].

**Intima–media thickness and carotid plaque**

With the patient in the supine position and the neck in partial extension, we have scanned right carotid artery through an ultrasonography device (Philips Sonos 5500). The transducer was manually oriented perpendicularly to the longitudinal axis of the vessel under B-mode guidance, and common carotid IMT was measured at a posterior wall site located 2 cm below bifurcation as the difference between the inner echoicogenic and the middle anechoicogenic layers.

In our laboratory, the intra-session within-operator and between-operator variability of IMT amounts respectively to a coefficient of variation of the mean value of 2.5% and to 2%, the corresponding value for the inter-session between-operator variability being 3.9%.

Carotid plaque was defined as the presence of a IMT more than 1.2 mm in the common carotid, bulb or internal carotid artery.

**Statistical analysis**

Data obtained in each patient were averaged, and individual data were summed and expressed as means (±SD), separately for the hypertension and control groups.

Between-group differences were assessed by Student t, Mann–Whitney and \( \chi^2 \) tests (or Fisher exact test when needed) for normally distributed, nonnormally distributed and categorical variables, respectively. Subgroup analysis was performed according to TOD status using one-way analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons.

Pearson’s or Spearman’s correlation coefficients were used, as appropriate, to test the association between variables. We performed linear regression using the additive model and adjusting for covariates determined by stepwise regression. We used PWV, LVM and IMT as the dependent variables with AnxA5, age, sex, SBP, waist circumference, TC, fasting blood glucose and the presence of diabetes, serum creatinine and antihypertensive treatment (in hypertension patients) as covariates for multivariate adjustments. Antihypertensive treatment was used both as single-drug class and grouped under pleiotropic anti-inflammatory property (angiotensin-converting enzyme–inhibitors with AT1 blockers vs all the other drugs class).

In correlation and linear regression analysis, AnxA5 was normalized by logarithmic transformation as it had shown distributions departed from normality due to positive skewness.

**RESULTS**

**Population characteristics**

As shown in Table 1, age was similar in controls and treated hypertension patients that showed higher SBP, DBP, waist circumference, BMI and triglycerides with lower LDL and HDL cholesterol. Instead, serum TC, serum creatinine, eGFR and fasting blood glucose were similar in the two groups. A small percentage of hypertension patients were diabetic (8.1%, all on treatment), whereas no diabetes was seen in the control group.

Hypertension patients present higher AnxA5 levels (13.9 ± 11.1 vs 10.1 ± 8.4 ng/ml, \( P < 0.001 \)) and, regarding TOD, higher arterial stiffness (PWV: 8.5 ± 1.8 vs 7.6 ± 1.5 m/s, \( P < 0.001 \)) and LVMI (121.7 ± 29.3 vs 113.5 ± 21.1 g/m², \( P < 0.05 \)) as compared with normotensive patients, whereas carotid IMT was superimposable between the two groups (Table 1).

Accordingly, a PWV higher than 10 m/s was more frequent in hypertension patients (19.5 vs 4.1%, \( P < 0.001 \)), LVH was shown by 60.1% of the hypertension patients compared with 58.8% of the controls group (\( P = \) NS), and, finally, carotid plaques presence was similar between the two groups (34.9 vs 30.6%, \( P = \) NS).

**Annexin A5 and target organ damage**

Figure 1 shows AnxA5 levels when patients were divided according to the presence or absence of TOD in both treated hypertension and controls group.

AnxA5 was higher in hypertension patients with and without increased stiffness compared with control patients, whereas it was statistically different only when hypertension patient with LVH were compared with controls with LVH and when hypertension patients without LVH were compared with controls with and without LVH (Fig. 1, panel b).

AnxA5 was significantly higher in hypertension patients without carotid plaque compared with control patients without plaque, but it doesn’t result statistically significant when the comparison was made between hypertension patients with and without carotid involvement (Fig. 1, panel c).
In all patients (hypertension and controls), we found a positive correlation between AnxA5 and PWV \((r = 0.13, P < 0.05, \text{Fig. 3})\) and DBP \((r = 0.15, P < 0.01)\). On the contrary, it doesn’t correlate with LVMI or IMT, and no correlation was seen when analysis was performed in hypertension and controls separately.

Regarding TOD, PWV correlates with age \((r = 0.24; P < 0.05)\), sex \((r = 0.14; P < 0.05)\), SBP \((r = 0.44; P < 0.05)\), DBP \((r = 0.34; P < 0.05)\) and AnxA5 \((r = 0.13; P < 0.05)\) in all patients, whereas only SBP and DBP remained when hypertension group was considered separately \((r = 0.43 \text{ and } 0.31, \text{respectively}; P < 0.05)\).

With a total \(r^2\) of 0.5, age, sex, SBP and AnxA5 are the independent predictors of PWV at the multivariate analysis, whereas only SBP survives when analysis was done in the hypertension patients group \((r^2 = 0.24)\).
LVMI had shown significant correlation with sex, SBP, DBP, diagnosis of diabetes mellitus, waist circumference, HDL cholesterol and fasting blood glucose levels in all patients ($r$ systematically $>0.20; P<0.05$), whereas it correlates only with sex ($r=0.23; P<0.05$), SBP ($r=0.28; P<0.05$), diagnosis of diabetes ($r=0.26; P<0.05$) and fasting blood glucose ($r=0.23; P<0.05$) when only hypertension patients were considered.

With a total $r^2$ of 0.26, sex, SBP and presence of diabetes are the independent predictors of LVMI at the multivariate analysis, whereas only fasting blood glucose levels remain when analysis was done in the hypertension patients group ($r^2 = 0.16$).

Finally, IMT correlates only with age both in all patients and in hypertension patients group ($r=0.30; P<0.05$). At the multivariate analysis, age was the only independent predictor of IMT with a $r^2$ of 0.13 in all patients and 0.10 in hypertension patients.

**DISCUSSION**

In our treated hypertensive population, we found increased AnxA5 levels in comparison with normotensive control patients. We didn’t find a strong connection between AnxA5 levels and advanced vascular and cardiac TOD: In fact also patients with hypertension but without target organ involvement had shown increased AnxA5 levels, whereas an increasing number of TOD were not associated with its parallel increase. However, we found an important correlation between AnxA5 and PWV that resists to linear regression analysis.

Hypertension is able to determine arterial and heart system stress by a variety of mechanism. Surely the volume
and pressure overload can directly exert negative effects both at cardiac and vascular levels and is able to activate biochemical process that can deteriorate organ function via the activation of matrix metalloproteinase (MMP) and its tissue inhibitors system [22] or tissue renin–angiotensin–aldosterone system [23].

Inflammation pathways activations are particularly important in arterial stiffness process by several mechanism [24]. Prolinflammatory citokines and cell adhesion molecules lead to reduced NO bioavailability, increased production of reactive oxygen species, vascular smooth muscle cell migration and increased MMP activation and matrix degradation. As a result, the vascular inflammation will increase fibrosis and impair endothelial vasodilatation that leads to the arterial stiffening process.

In our hypothesis AnxA5 is secreted early into the bloodstream of hypertension patients by endothelial cell (both coronary and systemic) as a mechanism of defense that try to palliate a potential proinflammatory and hypercoagulable state that will finally lead to cardiac and vascular TOD. According to our data, higher plasma levels of AnxA5 have also been detected in other conditions associated with inflammation and increased coagulation states such as sickle cell disease [25] and systemic lupus erythematosus (SLE) [26,27].

A second result of our study that needs to be discussed is the direct influence of AnxA5 on arterial stiffness. This relationship should be the epiphenomenon of the negative effects of inflammation on arterial functioning. An increased inflammation leads to AnxA5 increased release from the endothelium, as the expression of an initial damage of the arterial wall. This damage can be measured as increased arterial stiffness, or an increased PWV value.

AnxA5 was previously found to be associated with LVMI in hypertensive patients [14] but even more with ejection fraction and systolic dysfunction [14,28]. Accordingly, it has been shown a reduction in AnxA5 plasma levels after cardiac resynchronization therapy and increase in systolic function [15]. Although we didn’t find the correlation between AnxA5 and LVH, our study provides an additional piece of information on this topic. The reason of the different results of our study probably depends on the higher ejection fraction of our patients in comparison with those studied in the cited article (62 vs 54%) [14]. A previous plasma cutoff point of 24 ng/ml was determined for AnxA5 as a value discriminating severe from moderate systolic dysfunction in patients with heart failure and ventricular dyssynchrony at baseline, before treatment with cardiac resynchronization therapy [15]. On the other hand, the values of plasma AnxA5 reported in this study correspond to asymptomatic hypertensive and normotensive patients, and therefore, in a much less severe clinical status than patients with heart failure. In fact, our results confirm previous observations of plasma AnxA5 levels of ~13 ng/ml previously quantified in an independent cohort of hypertensive patients without symptoms of heart failure [24]. Of interest, values of AnxA5 determined in the emergency room in patients with ischemic cardiac damage are close to 16 ng/ml [28]. Therefore, the relevance of these findings should be interpreted in the sense that the difference observed in plasma AnxA5 between hypertensive and normotensive patients may be indicating early changes related with incipient vascular damage (as a protein highly expressed in the endothelium that associates with arterial stiffness in the current study), and that for AnxA5 to be an indicator and monitored cardiac damage, a more advance state of the cardiac disease is probably needed.

Regarding carotid involvement, AnxA5 has been found related to IMT in one study on SLE patients [16], whereas this association has not been confirmed in hypercholesterolemic patients [29]. However, SLE patients present a more pronounced inflammation than hypertension and hypercholesterolemic patients and this have to be taken in account when interpreting these data and is probably the explanation of the different results.

A final comment has to be done on an interesting issue, that is the AnxA5 haplotypes. In fact, little is known about the single nucleotide polymorphisms in AnxA5 in terms of their relative contribution to cardiovascular disease (CVD) risk so far. It has been described that the minor T-allele of rs1113129 in exon 2 of AnxA5 is associated with decreased risk of MI under the age of 45 years [30] and with a lower risk of a new thrombotic event during a 35-month follow-up [31]. Subsequent studies, however, were unable to replicate these findings [32]. AnxA5 M2 haplotype has been linked to thrombotic obstetric complications [33,34], although its contribution to CVD risk remains to be determined. In addition, the contribution of AnxA5 genetic variations to CVD risk in patients with familiar hypercholesterolemia has not been confirmed [27]. On the other hand, interesting results have been published concerning AnxA5 intronic SNPs rs4833229 and rs6830321 and their association with increased restenosis risk in patients undergoing percutaneous coronary intervention for atherosclerosis [35]. Therefore, the potential involvement of AnxA5 haplotypes in the increment of the cardiovascular risk seems to be relevant mainly in the atherosclerotic context, although with controversial results. Taking this into account, we did not consider measurement of AnxA5 haplotypes as a priority in our hypertensive patients, although this issue holds great interest and deserves to be approached in further studies.

Our study has some limitations: First, it had a cross-sectional nature, which means that it does not provide evidence on progression of the TOD and its association with AnxA5 levels. Second, we did not obtain a comprehensive assessment of cardiovascular risk profile and could thus not provide a description of the relationship of alterations found with cardiovascular risk factors. Finally, anti-hypertensive and lipid-lowering therapy can exert anti-inflammatory and pleiotropic effects. Although no effects of therapy have been found in multivariate analysis, it has to be highlighted that our patients are all treated, and so the results are not generalizable in untreated patients.

Our study, however, has also some element of strength. First, TOD has been measured by state-of-the-art techniques. Furthermore, our controls are blood donors free from any cardiovascular or metabolic overt disease, and thus a quite perfect control population to be used for comparisons.

In conclusion, the principal result of our work is thus that the AnxA5, secreted by endothelial cells, is increased in
treated hypertension patients, independently by the presence of TOD but rather by the presence of hypertension itself. In hypertensive patients, it is probably released in the plasma as a defensive mechanism through its anti-inflammatory and anticoagulant effects. Arterial stiffness seems to be associated with the levels of Annexin A5, whereas other classic hypertensive TODs do not.

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Conflicts of interest

There are no conflicts of interest.

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**Reviewers’ Summary Evaluation**

**Reviewer 1**

This is a nice paper where the authors tried to evaluate AnxA5 in treated hypertensive patients compared with normotensive controls to determine whether it is associated with target organ damage evaluated as arterial stiffness, carotid plaque and left ventricular hypertrophy. The methodology used could be considered as one of the strengths of this paper, as results are based on a careful examination of each patient. Nevertheless as the atherosclerotic process is a quite complex one, considering AnxA5 as an isolated factor is a limitation, but it is expected the authors will extend their investigation to other molecular mediators.