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Abstract

Granulomatosis with polyangiitis (GPA) is an autoimmune vasculitis associated with anti-neutrophil-cytoplasmic antibodies (ANCA) against proteinase 3 leading to kidney damage. Neutrophils from those patients have increased expression of membrane proteinase 3 during apoptosis. Here we examined whether neutrophils from patients with GPA have dysregulated protein expressions associated with apoptosis. A global proteomic analysis was performed comparing neutrophils from patients with GPA, with healthy individuals under basal conditions and during apoptosis. At disease onset, the cytosolic proteome of neutrophils of patients with GPA before treatment was significantly different from healthy controls and this dysregulation was more pronounced following *ex-vivo* apoptosis. Proteins involved in cell death/survival were altered in neutrophils of patients with GPA. Several proteins identified were PR3-binding partners involved in the clearance of apoptotic cells, namely calreticulin, annexin-A1 and phospholipid scramblase 1. These proteins form a platform at the membrane of apoptotic neutrophils in patients with GPA but not healthy individuals and this was associated with the clinical presentation of GPA. Thus, our study shows that neutrophils from patients with GPA have an intrinsic dysregulation in proteins involved in apoptotic cell clearance which could contribute to the unabated inflammation and autoimmunity in GPA. Hence, harnessing these dysregulated pathways could lead to novel biomarkers and targeted therapeutic opportunities to treat kidney disease.

Translational Statement

The cytosolic proteome in neutrophils from patients with Granulomatosis with polyangitiis (GPA) with active disease can be distinguished between healthy controls and patients in remission and display dysregulated death pathways. The interaction of the autoantigen PR3 with its partners such calreticulin, phospholipid scramblase 1 and annexin-A1 at the surface of apoptotic neutrophils may contribute to increased survival and pro-inflammatory characteristics of apoptotic neutrophils. Membrane expression of PR3 partners during apoptosis could represent new markers of disease activity in GPA and modulating their expression and/or activities could represent therapeutic perspective in GPA.

Introduction

Granulomatosis with Polyangiitis (GPA) is an autoimmune vasculitis characterized by anti-neutrophil cytoplasmic antibodies (ANCA) directed against proteinase 3 (PR3) and, less frequently, myeloperoxidase (MPO)[1]. While the pathogenesis of ANCA-associated vasculitides is poorly understood, neutrophils play a critical role as both the target of autoimmunity and the effector cells responsible for endothelial dysfunction within the microvasculature as well as kidney damages[2]. It has been shown that neutrophil apoptosis is delayed in GPA[3]. Indeed, tight control of neutrophil survival is required for effective elimination of dying cells before they release their cytotoxic content and feed-forward tissue damage. Furthermore, their clearance by macrophages is a key step in the eventual resolution of inflammation[4]. A delay or disturbance in apoptosis is one mechanism responsible for breaking tolerance in various autoimmune diseases including rheumatoid arthritis and systemic lupus erythematous[1, 5, 6]. Previous experiments have also suggested that these processes are altered in neutrophils from GPA patients[7-9] and that the autoantigen PR3 could be involved in this process[10]. Indeed, membrane expression of PR3 on apoptotic neutrophils is increased in GPA compared to healthy controls[11]. During this process PR3 is associated with partner proteins involved in the recognition and clearance of apoptotic cells such as calreticulin (CRT)[12], described as an "a eat-me signal", phospholipidscramblase 1 (PLSCR1)[13], an enzyme responsible for the movement of lipids across the plasma membrane and C1q, a bridging molecule involved in the clearance of apoptotic cells[14]. This interferes with this latter process resulting in a disruption of immune silencing, favoring autoimmunity and perpetuating inflammatory processes[15, 16]. Through its ability to bind the lipid phosphatidylserine (PS), PR3 can bind microvesicles or bystander apoptotic cells at the site of inflammation which in turn may modulate the inflammation[17]. Herein we provide evidence

of a disturbance in both apoptosis and the immune mechanisms associated with apoptotic cell clearance alterations in neutrophils from GPA patients amenable to therapeutic interventions.

Results

Apoptotic neutrophil from GPA patients show abnormal expression of cytosolic proteins.

We performed a proteomic analysis of the cytosol of neutrophils from GPA patients at disease onset in the absence of glucocorticoid treatment (GPA0) and during remission (GPA12) compared to healthy controls (HC) neutrophils at the basal state (B) and after apoptosis (A) (**Figure 1A**). A total of 96 proteins (**Supplementary Table S1**) were found as differentially expressed in one of the 9 different group comparisons between GPA0-B vs HC-B; GPA12-B vs HC-B; GPA12-B vs GPA0-B (**Figure 1B**); GPA0-A vs HC-A; GPA12-A vs HC-A; GPA12-A vs GPA0-A (**Figure 1C**); HC-A vs HC-B; GPA0-A vs GPA0-B; GPA12-A vs GPA12-B (**Figure 1D**). Principal component analysis was performed on the proteins of interest and showed four different clusters of cytosolic proteomes that correspond to the four following groups of neutrophils, namely HC-B, GPA0-B, HC-A and GPA0-A (**Figure 1A**) which demonstrate that the cytosolic proteome of neutrophils is significantly different between HC and GPA0 at disease onset both in the basal and in the apoptotic state. The percentage of variation accounted for by the first component is 68.3% and 10.4% for the second component. Notably, the neutrophil cytosols corresponding to the GPA patients in remission (GPA12) were in an intermediate position between HC and GPA0 both at the basal state and after apoptosis strongly suggesting that treatment significantly shifted the cytosolic proteome of GPA towards HC. The Venn diagram (**Figure 1B)** shows the numbers of spots that are different under the basal state between the 3 groups of donors including HC, GPA0 and GPA12. It shows that there are 34 different spots between HC and GPA at onset whereas there are only 23 different spots when GPA patients have been treated thus showing that the

treatment can minimize the differences between HC and GPA. Accordingly, we found 19 spots differentially expressed in GPA patients before and after treatment. Similar analysis was performed in neutrophils after apoptosis (**Figure 1C**) and shows that the number of different spots between HC and GPA0 is 46 whereas it is only 20 between HC and GPA12 confirming the effect of treatment on neutrophil proteome. This was confirmed by a decreased number of spots that are modified during apoptosis (**Figure 1D**) in HC (71) versus GPA0 (39) confirming a defect in the modulation of proteins involved in apoptosis-related pathways. This defect was partially normalized in GPA after treatment with only 52 spots modified by apoptosis.

Similar principal component analysis was performed in a subgroup of MPA patients which allowed to differentiate cytosolic proteome patterns between GPA and MPA after apoptosis (**Supplementary Figure S2**) suggesting that molecular disturbances of apoptotic neutrophils differ between GPA and MPA.

Identification of proteins differentially expressed between HC and GPA0 under basal or apoptotic (**Supplementary Table S2-S3**) showed that they were predominantly involved in cell death and survival, cell movement and carbohydrate metabolism (**Supplementary Figure S1B**).

As an example, one of those proteins differentially expressed between HC and GPA during apoptosis was identified as S100A9 (**Supplementary Figure S3**). It is predominantly found as calprotectin (S100A8/A9 heterodimer) and is a promising biomarker in inflammatory diseases including rheumatoid arthritis[18] and GPA[19, 20].

Ingenuity Pathway Analysis was used to visualize the biological connections between proteins identified as dysregulated in GPA (**Figure 2A**). Importantly, various known PR3 binding partners were differentially regulated during neutrophil apoptosis in GPA compared with HC, including CRT[12] and annexin-A1 (AnxA1)[21]. Taken together, the proteomic

analysis indicated a dysregulation of the PR3-related pathways within the cytosol of apoptotic neutrophils from GPA patients and as such specific analysis were performed on these PR3 partners, including AnxA1, a protein implicated in neutrophil apoptosis and the resolution of inflammation. Indeed, the proteomics analysis confirmed that some spots corresponding to AnxA1 decreased upon apoptosis in HC as well as GPA (**Figure 2B**). While no differences in AnxA1 expression at 37kDa was observed under basal conditions, apoptotic GPA neutrophils had a lower decrease in AnxA1 compared with HC cells as shown by Western blot analysis (**Figure 2C-D**). Of note, we confirmed that AnxA1 is cleaved during apoptosis[22-24] (**Supplementary Figure S4**).

Analysis of activation and apoptosis markers in neutrophils: increased membrane expression of AnxA1 after apoptosis

We previously reported that spontaneous apoptosis of neutrophils from GPA is diminished compared with HC[3, 16]. Accordingly, membrane CD16 which is downregulated during apoptosis[25] (**Figure 3A**) was significantly increased in apoptotic neutrophils from GPA patients at diagnosis compared to HC, further supporting the notion that neutrophils from patients have altered apoptosis. CD16 was also significantly decreased in neutrophils from patients in remission, as quantified both at resting state and after spontaneous apoptosis. No significant differences between the membrane expression of CD11b[26] or CCR5[27] (**Supplementary Figure S5)** used as markers of neutrophil activation or apoptosis, respectively between HC and GPA at onset or after remission were observed. We observed a significant increase in the percentage of apoptotic neutrophils expressing membrane PR3 in GPA at diagnosis but also during remission compared with HC (**Figure 3B**) thus suggesting that current treatment did not decrease PR3 expression. While apoptosis significantly increased AnxA1 membrane expression, there was no significant difference between HC and

GPA patients at diagnosis or remission. (**Figure 3C**). AnxA1 is a PS binding protein could be a pivotal scaffolding piece in the apoptosis induced membrane PR3-associated protein platform by interacting directly with PR3. Surface plasmon resonance (SPR) experiments were performed to examine the direct interaction between PR3 and AnxA1. Injection of various concentrations of AnxA1 over immobilized PR3 allowed the determination of a K_D value of 5.7×10^{-6}M which is indicative of a potential interaction at the membrane of neutrophils (**Figure 3D**). More importantly, there was a significant positive correlation between membrane PR3 and AnxA1 in resting and apoptotic neutrophils in GPA patients at diagnosis (**Figure 4B-D**) but not in HC cells (**Figure 4A-C**). Serum levels of AnxA1 were unaltered across the three groups (data not shown) irrespective of treatment, indicating that AnxA1 may act as a key element in the intrinsic defect of neutrophils rather a systemic mediator in this disease. AnxA1 has been shown to be released from dying neutrophils[22-24] and has a role in apoptotic cell clearance. Treatment of apoptotic neutrophils with exogenous AnxA1 increased the proportion of neutrophils expressing membrane PR3 only in HC but not in GPA patients. Notably, neutrophils from GPA patients during remission were able to externalize PR3 in response to AnxA1 treatment at similar levels than HC (**Supplementary Figure S6**). All together our data suggest that the apoptosis induced membrane PR3-associated protein platform which includes AnxA1 at the surface of apoptotic neutrophils is dysregulated in GPA at the disease onset.

PLSCR1 membrane expression in apoptotic neutrophils from GPA patients correlates with AnxA1. We have previously described that PR3 is co-externalized with PS and PLSCR1, a protein involved in the lipid redistribution during cell apoptosis and activation[13]. There was a small number neutrophils expressing membrane PLSCR1 under basal conditions and no difference was observed between HC and GPA patients at diagnosis or following treatment (**Figure 5A**). While apoptosis increased PLSCR1 membrane expression in all groups, there

was a marked upregulation of membrane PLSCR1 on a subset apoptotic neutrophils from GPA patients at diagnosis compared with both HC and GPA at remission. SPR analysis was used to further characterize the interaction between PLSR1 and PR3 and provided evidence that immobilized PR3 associated with PLSCR1 with a high affinity K_D of 2.18×10^{-8}M (**Figure 5B)**. Accordingly, we observed a significant correlation between membrane expression of PR3 and PLSCR1 in basal and apoptotic neutrophils from GPA patients at diagnosis (**Figure 5D-5F**) whereas no correlation was observed in HC (**Figure 5C-5E)** or in GPA in remission (data not shown). There was also a significant correlation between the externalization of AnxA1 and PLSCR1 in apoptotic neutrophils from GPA patients and again not in HC (**Figure 5H-G**) although no correlation was observed in the basal state (data not shown). SPR analysis confirmed that immobilized PLSCR1 could interact with AnxA1 with a rather strong affinity constant represented by a K_D of 5.2×10^{-7}M (**Figure 6A)**. The colocalization of AnxA1 and PLSCR1 was confirmed by indirect immunofluorescence on apoptotic but not on resting neutrophils from HC suggesting that this association only takes place during apoptosis.

We further confirmed the interaction between PR3, PLSCR1 and AnxA1 at the cellular level using apoptotic RBL cells transfected with either a control or PR3 expressing plasmid. We previously described that PR3 and CRT were associated at the membrane of apoptotic RBL cells[12, 28,29] (**Supplementary Figure S7**). These results suggest that the surface expressions of PR3, PLSCR1 and AnxA1 are functionally linked and that these three elements build the apoptosis induced membrane PR3-associated protein platform at the surface of apoptotic neutrophils in line with what was observed in GPA neutrophils.

Membrane PR3-associated protein platform is modulated by the clinical presentation of GPA

In a better attempt to correlate our findings with clinical symptomatology, we analyzed the association between BVAS and each neutrophil parameter we quantified, including membrane expression of CD16, CD11b, CCR5, PR3, AnxA1 and PLSCR1 under basal state and after apoptosis. No correlation between BVAS and neutrophil membrane proteins was found when all the GPA patients were included.

Next, we classified the patients according their clinical symptomatology into 2 groups: Group#1 included patients with lung and ENT signs but without renal involvement (n=16) whereas Group#2 included patients with biopsy-proven kidney disease (with or without ENT) (n=30). With this analysis, we revealed a significant difference in BVAS between the Group#1 (10.31±1.61) versus Group#2 (18.37±1.31) (Student-*t* test, P=0.0005) thus confirming that Group#2 bore a more severe degree of vasculitis compared to Group#1 (**Figure 7A**). Nonetheless, no correlation between BVAS and PR3 membrane expression on apoptotic neutrophils was observed neither in Group#1 nor in Group#2. Interestingly, when the GPA patients were subdivided according to their symptomatology, the correlation between the membrane expression of AnxA1 and PR3 on apoptotic neutrophils was observed only in the Group#2 (P=0.004, R^2=0.316) (**Figure 7B**) at onset and was not observed after remission (data not shown). These data suggest that apoptosis-induced PR3 membrane expression and its related partners especially AnxA1 might participate to the pathophysiological mechanisms involved in vasculitis with renal involvement.

Discussion

While the mechanisms contributing to the pathogenesis of GPA remain largely unknown, neutrophils play a key role and understanding how these cells are dysregulated and identifying specific pathways altered is essential for the development of targeted therapeutics.

There is strong evidence to suggest that neutrophil apoptosis and clearance of apoptotic neutrophils by macrophages are disturbed in GPA. Neutrophils from GPA patients exhibit increased survival compared to HC and apoptotic neutrophils from patients express more PR3 autoantigen on their surface[3, 11, 16]. Not only can PR3 on apoptotic neutrophils interfere with their clearance by macrophages[12, 30], membrane PR3 also subverts their normal anti-inflammatory reprogramming and triggers the synthesis of pro-inflammatory cytokines[16]. It is with this in mind that we examined the proteome of neutrophils from HC and GPA at disease onset and during remission. Our global proteomic approach demonstrates that even under basal conditions, neutrophils from GPA patients at onset are fundamentally different from HC and these differences decrease at remission. Despite this, even in remission, proteomic results from GPA neutrophils do not fully resemble HC indicating that treatment could not overcome all intrinsic defects likely to contribute to the pathogenesis of this disease. Interestingly, our proteomics analysis of neutrophil cytosols under basal conditions has identified S100A8/A9 which constitute 40% of cytosolic proteins, a disease biomarker recently identified in GPA[20, 31] demonstrating that examining dysregulated cytosolic proteins could represent a valid tool for biomarker discovery. Notably, S100A8/9 proteins complexes which are stabilized and secreted during apoptosis can promote neutrophil survival thus potentially delaying the resolution of inflammation[32].

However, the most remarkable differences between neutrophils from HC and GPA neutrophil cytosols were found when cells entered apoptosis compared to basally. Many of the proteins identified were involved in cell survival/death, supporting our hypothesis that neutrophils from GPA have intrinsic defects in apoptotic pathways. Notably, one limitation of our study is that it relies on *ex vivo* analysis of spontaneous apoptosis which might be different from neutrophil death *in vivo* at the inflammatory site. In addition, neutrophil death mechanisms

are much more complex, depending on the type of inflammation and can greatly affect the disease outcome.

Various proteins dysregulated in GPA were known PR3 binding partners, indicating a central role for the autoantigen in the pathogenesis of GPA. One PR3 partner protein identified in this study was AnxA1, a member of the Annexin superfamily of Ca^{2+} and phospholipid binding protein[33]. AnxA1 relocates to the cell membrane during activation or apoptosis[34] and when expressed on apoptotic cells, facilitates their recognition and clearance by macrophages[22, 34, 35]. Here we report that cytosolic AnxA1 is decreased during apoptosis and is translocated at the membrane of the neutrophils where it associates with PR3 and PLSCR1, a plasma membrane protein that participates in the PS externalization to the outer membrane during apoptosis[36]. Little is known about the role of PLSCR1 in neutrophil function and more broadly in the regulation of inflammation[37]. We have previously described that PR3 is functionally associated with PLSCR1 and CRT at the membrane of apoptotic neutrophils suggesting a potential role for this protein association in the clearance of apoptotic neutrophils[13]. Notably, there was a strong upregulation of PLSCR1 on the surface of apoptotic GPA neutrophils during disease onset compared with HC, while after treatment values returned to normal. The formation of this apoptosis-induced membrane PR3-associated protein platform was next confirmed in RBL cells transfected with PR3. This cellular model has been invaluable to study PR3 activity, membrane anchorage[28, 29] and its association with CRT[12]. Most strikingly, SPR analysis has demonstrated unambiguously the individual interactions between PR3 and AnxA1, PLSCR1 or CRT[12]. Furthermore, it was previously shown that PR3 like AnxA1 can bind PS[17] which is known to interact with CRT[38]. However, no direct interaction was found between AnxA1 and CRT strongly suggesting that

the formation of this specific molecular reorganization is regulated at the surface of apoptotic neutrophils in which PR3 plays a central role.

Most strikingly, we observed that in GPA patients and not HC, PR3 membrane expression was tightly correlated with surface expression of both AnxA1 and PLSCR1 strongly suggesting that the expression of these proteins are closely related. Interestingly, while treatment of HC neutrophils with exogenous AnxA1 during apoptosis upregulated membrane PR3, this upregulation was not observed in GPA neutrophils at diagnosis. This result was not due to a difference in the ability of exogenous AnxA1 to bind to neutrophils (data not shown) or expression of the AnxA1 receptor, FPR2[39] as this receptor was similarly expressed in HC and GPA patients (data not shown). While the mechanisms responsible for this need further investigation, the results clearly indicate that exogenous AnxA1 does not produce the same effects in apoptotic neutrophils from GPA patients compared with HC. Pertinently, the correlation between PR3 and AnxA1 at the membrane of apoptotic neutrophils in GPA was associated with the clinical symptomatology of GPA patients and the correlation was more significant in patients with more severe disease corresponding to patients with renal involvement. Our current findings suggest that PR3 membrane expression is modulated by disease severity[40-42], which might be associated with specific disturbances in neutrophil activation and death mechanisms.

With regard with the clinical specificity of our findings, our proteomic analysis performed in neutrophils from untreated MPA patients at the onset of disease have shown that we can differentiate between the cytosol from patient with GPA and MPA both at the basal state and after apoptosis. For instance, no significant difference was found in the level of AnxA1 or CRT in the cytosols from MPA patients. Notably, we observed an increased in S100A8/A9 in cytosol from MPA similarly to what we observed in GPA patients. This suggests the existence of molecular alterations that are disease specific but this issue would require further

experiments. Pertinently, recent reports showed a dysregulated synthesis of PR3 in mature neutrophils in GPA[43], which may account for the specific localization and function of PR3 observed in the current study. In addition, an epigenetic defect in the control of PR3 expression has been described[44] which has been associated with both the clinical status and relapse in GPA patients[45].

Consistent with this notion that PR3 acts as a trigger for neutrophil dysregulation in GPA, we suggest that the epigenetic control of expression and synthesis of PR3 partners like AnxA1 or PLSCR1 may also be intrinsically modified in GPA neutrophils. The fact that the cytosolic proteome in neutrophils from GPA patients with active disease can be easily distinguished between HC and patients in remission indicates that our proteomic approach is valuable in identifying not only new markers of disease activity but also potential therapeutic opportunities[46] and especially targeting serine proteinase activities[47].

Material and Methods

Study approval and clinical samples

Healthy control (HC) donors and GPA patients gave written informed consent to participate in NeutroVasc study (#2010-AOM10055) approved by the INSERM Institutional Review Board (NEUTROVASC-DR-2012-002) and the Cochin Hospital Ethics Committee according to the Declaration of Helsinki (CPP authorization #2011-12797).

HC were recruited at the Etablissement Français du Sang (EFS, Paris). GPA patients had to fulfill classification criteria of the American College of Rheumatology[48] and the definition from the 2012 Chapel Hill Consensus Conference for GPA[49] and those at onset of disease a BVAS (Birmingham Vasculitis Activity Score) greater than 3 (Supplementary Table S4). Neutrophils were analyzed both at onset of disease (GPA0) and at remission (12 months after diagnosis, GPA12). Sera were collected from GPA patients and HC and stored at -80°C until measurement of AnxA1 (Elabscience, Chicago-IL) and S100A8/9 (Thermofisher) by ELISA kit.

Neutrophil experiments

Neutrophils from GPA and HC donors were isolated from EDTA-anticoagulated blood using density-gradient centrifugation on Ficoll®[12]. To induce physiological apoptosis, 2×10^6 neutrophils/ml were resuspended in RPMI-1640 with 10%FCS and incubated for 16h at 37°C. To measure apoptosis, cells were labeled with Annexin-V and 7-AAD to assess apoptotic versus necrotic cells, respectively[5]. When indicated neutrophils under basal conditions, after activation (TNFα 5ng/ml, 15min at 37°C) or apoptosis (16h at 37°C) were treated with recombinant AnxA1 (20µg/ml) as previously described[50] and PR3 or AnxA1 membrane expression was measured.

2D-DIGE comparative proteomic analysis and mass spectrometry

Neutrophil cytosolic fractions were obtained following low-level sonication as previously described (1Hz for 10s)[51]. Neutrophil cytosols were analyzed using 2D-DIGE (Supplementary Materials) and Ingenuity pathway analysis was used to analyse protein networks (https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis).

Flow cytometry analysis

Expression of membrane PR3, CD16, CD11b, CCR5, S100A8/9, AxA1 and PLSCR1 were performed and analyzed on a BD Accuri (BD Biosciences, San Jose, CA) as previously described[5] (Supplementary Materials). For apoptotic neutrophil analysis, a gating strategy on annexin-V-positive/7AAD-negative cells was used.

Western blot analysis

Samples were separated on a 10%SDS-PAGE and transferred to PVDF membranes (Biorad) as previously described[12, 13] and the primary antibodies were rabbit anti-AnxA1 (Invitrogen), S100A9 (Epitomics) and goat anti-β-actin (I-19, Santa Cruz-Biotechnology). Blots were developed using Chemiluminescent Substrate (ThermoScientific, Waltham-MA). Quantification of bands was performed using ImageJ software (NIH) where AnxA1 was normalized to actin.

Surface plasmon resonance (SPR) analysis

SPR measurements were performed using a BIAcore system (GE Healthcare) designed to measure the direct interaction between purified PR3, recombinant AnxA1[50], CRT[38] and PLSCR1[52] as previously reported[12,14] and to calculate affinity parameters (Supplementary Materials).

Labelling of AnxA1 and PLSCR1 using indirect immunofluorescence

Neutrophils either at basal state or after apoptosis were induced to adhere on coverslips and were labeled as previously described[14] using mouse anti-AnxA1 and goat anti-PLSCR1 (SantaCruz) antibodies (Supplementary Materials). Cells were visualized under a laser spinning-disk confocal microscope.

Statistical analysis

Data are mean±SEM. Statistical analysis was performed with Prism Software (GraphPad). Comparisons were made using a one-way ANOVA or Student *t* test or Mann Whitney U test where appropriate. Correlations were assessed using Pearson's test. Statistical significance was established at $P<0.05$.

Disclosure*:* The authors have no conflicting financial interests.

Author contributions:

JEG, KRM, JM, AR, PC, MLG performed experiments; CR purified AnxA1; PTD, PF performed SPR; LM organized the clinical study; JMN, BB, AK, XP, BT, LG recruited patients/analyzed results. JEG, KRM, PC, MLG, MP, PF, NT participated to discussion/writing. VWS conceived/wrote the project. All authors approved the manuscript.

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Supplementary Information

Supplementary information is available at Kidney International's website

Supplementary Materials ad Methods

Supplementary Figures:

Figure S1: Principal component analysis of the 96 proteins differentially expressed identified by Mass Spectrometry MALDI-TOF

Figure S2: Proteomic analysis of the neutrophil cytosol detects significant differences between GPA, MPA (M) and healthy controls (HC) under basal and apoptosis conditions

Figure S3: S100A8/9 protein is increased in the cytosol of neutrophils during apoptosis and serum levels in GPA were associated with the disease state.

Figure S4: AnxA1 protein is cleaved in the cytosol of neutrophils during apoptosis

Figure S5: Membrane expression of CD11b and CCR5 on neutrophils from HC and GPA patients at onset (GPA0) and after remission (GPA12)

Figure S6: Percentage of neutrophils expressing membrane PR3 following treatment with exogenous AnxA1 at the basal state, during activation and during apoptosis

Figure S7: Concomitant externalization of PR3, AnxA1 and PLSCR1 during apoptosis of Rat Basophilic Leukemia (RBL) cells

Supplementary Tables:

Table S1: Complete list of spots protein of interest obtained by 2D-DIGE analysis

Table S2: Proteins spots differentially expressed and identified by mass spectrometry of neutrophils from GPA patients and HC in basal state

Table S3: Proteins spots differentially expressed and identified by mass spectrometry of neutrophils from GPA patients and HC in apoptotic state

Table S4: Clinical characteristics of patients included in the neutrovasc protocol

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Figure Legends

Figure 1. Proteomic analysis of the neutrophil cytosol detects significant differences between GPA and HC cells under basal and apoptosis conditions. Six different conditions were analyzed: Cytosolic proteome of neutrophils from HC in basal and apoptotic state (HC B; n=6) and HC A; n=6), from GPA patients at onset of disease under in basal and apoptotic state (GPA 0 B; n=4 and GPA 0 A; n=4) as well as GPA patients under remission in basal and apoptotic state (GPA 12 B; n=3 and GPA 12 A; n=3). (**A**) Principal component analysis of the 96 proteins differentially expressed between GPA (at onset and remission) and HC, both in basal and apoptotic anlyzed by DeCyder^{TM} v7.0 EDA software module. A, Apoptosis, B: Basal, GPA 0: GPA patient at diagnosis, GPA 12: GPA patient at remission, HC: Healthy donor. (**B-D**) Pattern of differentially expressed proteins in basal and apoptotic neutrophils from HC, GPA at diagnosis (GPA 0) and at remission (GPA 12) shown as Venn diagrams. The intersections show the number of overlapped differentially expressed proteins in each area of the Venn diagram in neutrophils from GPA patients versus HC, respectively, in basal state and following apoptosis (*P*-value < 0.05).

Figure 2. Modulation of cytosolic AnxA1 expression during neutrophil apoptosis in neutrophils from GPA patients compared to HC. (**A**) Schematic network of identified proteins implicated in "cell death and survival" which are differentially expressed between apoptotic GPA and HC cells. Solid lines indicate direct interactions. Dashed lines indicate indirect interactions. Arrows indicate stimulation. AnxA1 is represented in red. ANP32A: Acidic leucine-rich nuclear phosphoprotein 32 family member A, ANXA1: Annexin A1, CALM1: Calmodulin, CALR: Calreticulin, CASP3: Caspase 3, GPI: Glucose-6-Phosphate Isomerase, HSPA5: Heat Shock Protein Family A (Hsp70) Member 5, LCN2: Lipocalin 2, LCP1: Lymphocyte Cytosolic Protein, P4HB: prolyl 4-hydroxylase, β polypeptide, also

known as PDIA1, PDIA3: protein disulfide isomerase family A, member 3, also known as ERp57, PLSCR1: Phospholipid Scramblase 1, PRTN3: Proteinase 3, SERBINB1; Serpin Family B Member 1, SET: Protein SET, SOD2: Superoxide dismutase, VCL: Vinculin. (**B**) Cytosolic AnxA1 abundance as measured using proteomics analysis in basal and apoptotic neutrophils from HC, GPA at diagnosis (GPA 0) and at remission (GPA 12, n = 4). (**C**-**D**) Western blot and densitometry analysis of full length AnxA1 in the cytosol of neutrophils in the basal state or following apoptosis from HC and GPA cells at diagnosis and during remission (n = 4 for each group). Notably, an additional band of AnxA1 could be observed at 29 kDa during apoptosis in both HC and GPA. All results were normalized to α-actin. Values are presented as mean ± SEM. Significant differences between groups were determined using a one-way ANOVA (C and D), **P* <0.05.

Figure 3. Membrane expression of CD16, PR3 and AnxA1 on neutrophils from HC and GPA patients. (A) Proportion of $CD16^+$ neutrophils under basal conditions and during apoptosis in healthy controls (HC, n = 33), GPA patients at onset (GPA 0, n = 20) and at remission (GPA 12, n = 14). Values are presented as mean ± SEM. (**B**). Proportion of apoptotic neutrophils expressing membrane PR3 from healthy controls (HC, n = 33), GPA at diagnosis (GPA 0, n = 38) and during remission (GPA 12, n = 30). Individual values are represented as dots. (**C**) Expression of membrane AnxA1 on neutrophils under basal or apoptotic conditions (Annexin V^+7AAD^-) from healthy controls (HC, n = 38), GPA patients at diagnosis (GPA 0, n = 18) and during remission (GPA 12, n = 17). Values are presented as mean ± SEM. (**D**) Analysis of PR3 interactions with AnxA1 measured by SPR spectroscopy. AnxA1 was injected at indicated concentrations over immobilized PR3 (1080 RU) in 10 mM HEPES, 145 mM NaCl, 2 mM $CaCl_2$, pH 7.4, and 0.05% surfactant P20 at a flow rate of 30 µl/min using a BIAcore T200 apparatus. Determination of the K_D performed by steady-state

affinity fitting was 5.7 x 10^{-6} M. Association curves were recorded for 120 s and dissociation for 120 s. The specific binding signals shown were obtained by subtracting the background signal over a reference surface with no protein immobilized and further subtraction of buffer blank injection. Data are representative of 3 experiments. All other conditions are described in section "Materials and Methods." RU, resonance unit. Significant differences between groups were determined using one-way ANOVA (A-D), $^{\#}$ or $^{*}P$ <0.05, $^{\#\#}$ or $^{**}P$ <0.01.

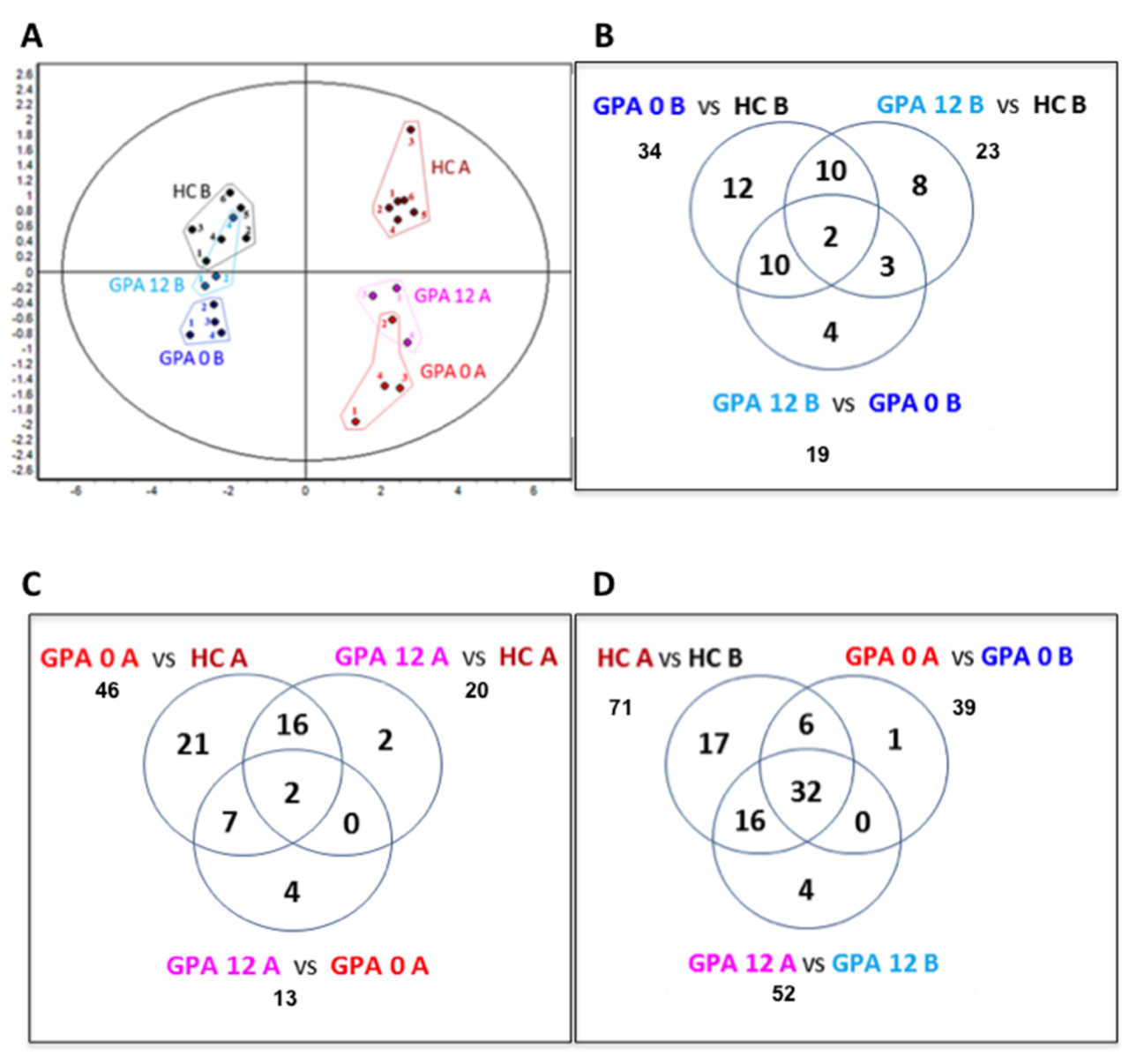
Figure 4. Correlation of AnxA1 membrane expression with membrane PR3 during apoptosis only in neutrophils from GPA patients but not HC. (A-D) Correlation between membrane expression of PR3 and AnxA1 MFI in neutrophils in the basal state (A and B) and during apoptosis (C and D) in HC and GPA patients at diagnosis.

Figure 5. Elevated PLSCR1 membrane expression during apoptosis and correlation with membrane PR3 or AnxA1 expression in neutrophils from GPA patients but not HC. (**A**) Proportion and extent of membrane PLSCR1 expression on neutrophils under basal and apoptotic conditions (Annexin V^+ $7AAD^-$) from healthy controls (HC, n = 25), GPA patients at diagnosis (GPA 0, n = 25) and during remission (GPA 12, n = 19). (**B**) Analysis of PR3 interactions with PLSRC1 measured by SPR spectroscopy. PLSCR1 was injected over immobilized PR3 (3500 RU) at increasing concentrations, as indicated, in 10 mM HEPES, 145 mM NaCl, 2 mM $CaCl_2$, pH 7.4, and 0.005% surfactant P20 at a flow rate of 20 µl/min using a BIAcore X apparatus. The dissociation constant K_D was 2.18 x 10^{-8} M. Association curves were recorded for 120 s and dissociation for 160 s. Data are representative of 3 experiments, RU: resonance unit. **(C-F)** Correlation between the degree of membrane expression of PR3 and PLSCR1 (MFI units) in neutrophils at basal state (B and C) and during apoptosis (D and E) in HC and GPA cells at diagnosis. **(G-H)** Correlation

between the extent of membrane expression of AnxA1 and PLSCR1 (MFI units) in HC and GPA neutrophils during apoptosis. Values in A are mean ± SEM. Significant differences between groups were determined using ANOVA (A) and correlations were assessed using Pearson's tests (B-E), *P <0.05, **P <0.01.

Figure 6. Interaction of AnxA1 and PLSCR1 measured by SPR and colocalization of both proteins in apoptotic neutrophils. (A) Interaction of the AnxA1 and PLSCR 1 analyzed by SPR spectroscopy. AnxA1 at indicated concentrations was injected over immobilized PLSRC1 (1100 RU) in 10 mM HEPES, 145 mM NaCl, 2 mM $CaCl_2$, pH 7.4, and 0.05% surfactant P20 at a flow rate of 30 µl/min using a BIAcore T200 apparatus. The specific binding signals shown were obtained by subtracting the background signal over a reference surface with no protein immobilized and further subtraction of buffer blank injection. Association curves were recorded for 120 s and dissociation for 160 s. The K_D value of 5.2 x 10-7 M was determined by steady-state affinity fitting. Data are representative of 3 experiments. RU, resonance unit**.** (**B**) Freshly isolated or apoptotic neutrophils were double labeled using an anti-AnxA1 mAb followed by a cy3-conjugated secondary antibody and an anti-PLSCR1 mAb followed by a Dylight405-conjugated secondary antibody as described in Materials and Methods. Samples were visualized by confocal microscopy under differential interference contrast (DIC) and emission filters adapted to Dylight405 and Cy3. Cy3 and Dylight405 fluorescences are represented in red and green respectively. Merge is shown (as indicated). Higher magnification is shown for the cell pointed by a white arrow. A scatterplot of red and green pixel intensities collected in the indicated region is represented. Scale bar 10 µm. Colocalization (yellow regions in merge) was evaluated by Pearson's correlation coefficient ≥0.8.

Figure 7. Correlation of membrane proteins with clinical symptomatology in neutrophils from GPA patients with or without renal involvement. (**A**) Comparison of BVAS from GPA patients at diagnosis without (Group #1, n = 16) or with (Group#2, n = 30) renal involvement **(B)** Correlation between membrane expression of PR3 and AnxA1 in apoptotic neutrophils from GPA patients from Group #1 (left) and Group #2 (right). Significant differences between groups were determined using Student t test (A) and correlations were assessed using Pearson's tests (B), $*P < 0.05$, $**P < 0.01$.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HC B
HC A
GPA 12 B
GPA 12 A
GPA 0 B
GPA 0 A
B
GPA 0 B vs HC B
GPA 12 B vs HC B
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12
10
8
2
10
3
4
GPA 12 B vs GPA 0 B
19
C
GPA 0 A vs HC A
GPA 12 A vs HC A
46
20
21
16
2
2
7
0
4
GPA 12 A vs GPA 0 A
13
D
HC A vs HC B
GPA 0 A vs GPA 0 B
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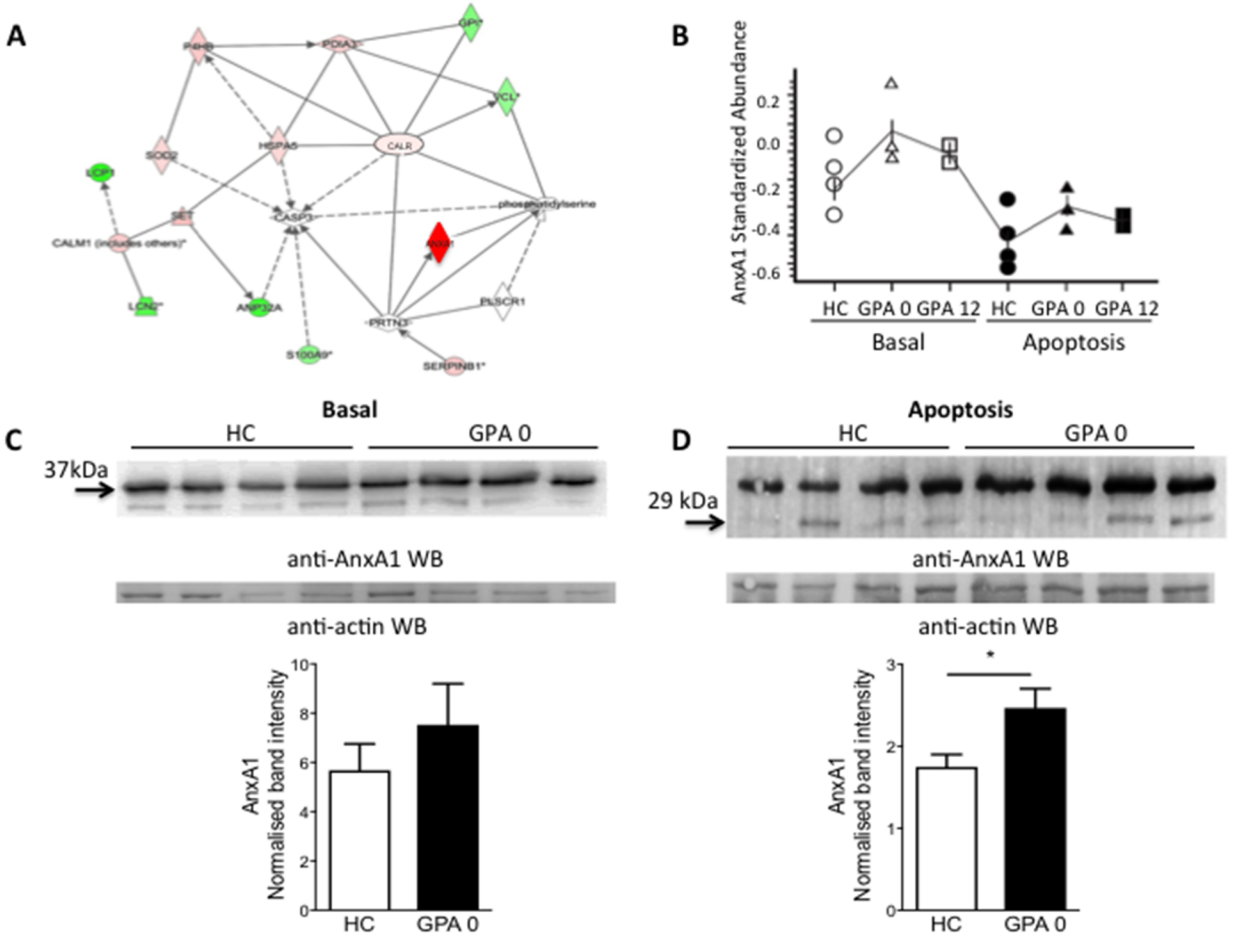
39
17
6
1
32
16
0
4
GPA 12 A vs GPA 12 B
52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GP*
PDIA3
PRDX6
VCL*
HSP90B1
HSP90AB1
SOD2
CALR
ICAM1
CASP3
phosphatidylserine
CALM1 (includes others)*
ANXA1
LCN2*
ANP32A
ELSCR1
PRTN3
S100A9*
SERPINB1*
B
AnxA1 Standardized Abundance
0.2
0.0
-0.2
-0.4
-0.6
HC
GPA 0
GPA 12
HC
GPA 0
GPA 12
Basal
Apoptosis
C
Basal
HC
GPA 0
37kDa
anti-AnxA1 WB
anti-actin WB
AnxA1
Normalised band intensity
10
8
6
4
2
0
HC
GPA 0
D
Apoptosis
HC
GPA 0
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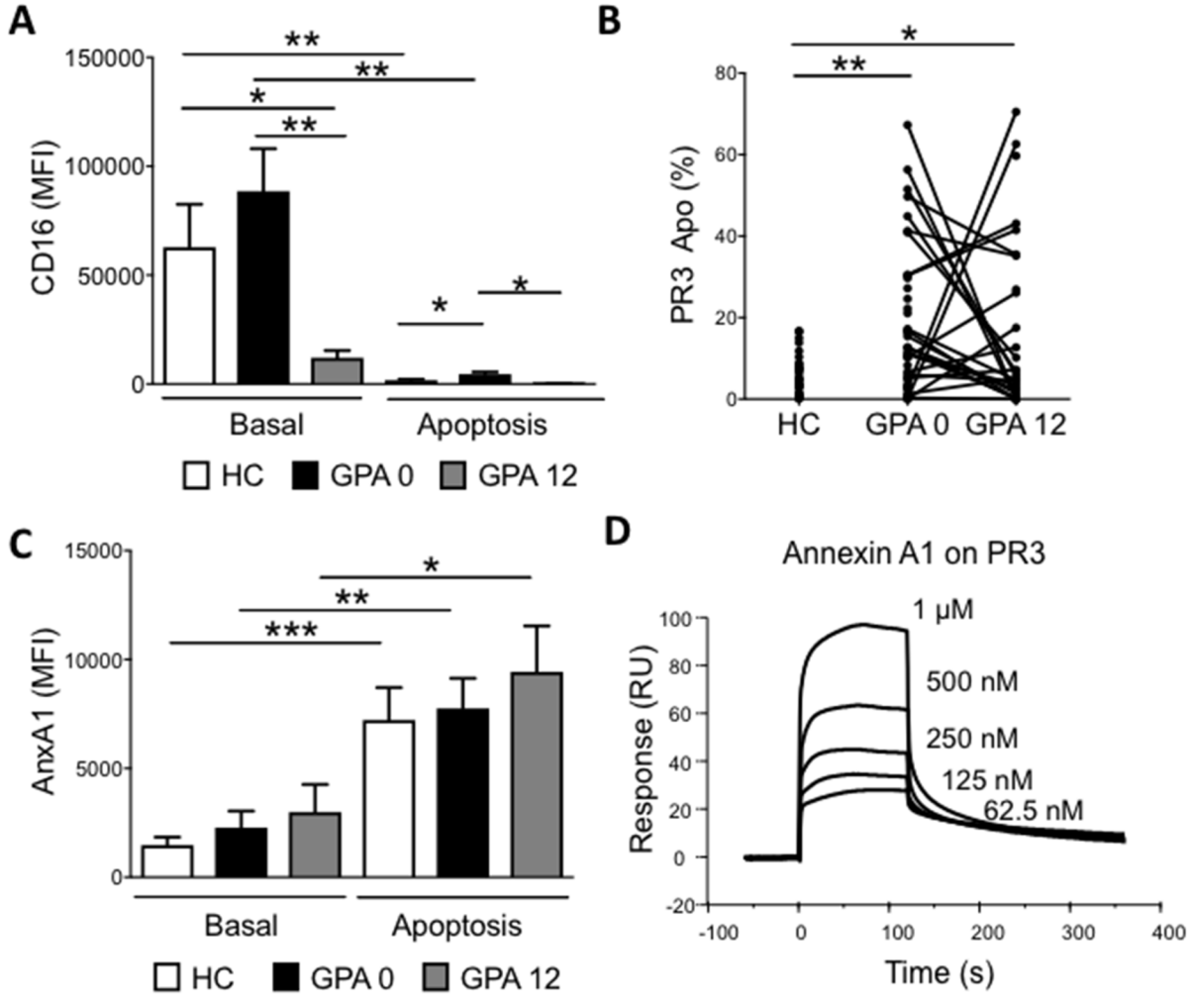
anti-AnxA1 WB
anti-actin WB
*
AnxA1
Normalised band intensity
3
2
1
0
HC
GPA 0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CD16 (MFI)
150000
100000
50000
0
**
**
*
**
*
*
Basal
Apoptosis
HC
GPA 0
GPA 12
B
PR3 Apo (%)
80
60
40
20
0
*
**
HC
GPA 0
GPA 12
C
AnxA1 (MFI)
15000
10000
5000
0
***
**
*
Basal
Apoptosis
HC
GPA 0
GPA 12
D
Annexin A1 on PR3
Response (RU)
100
80
60
40
20
0
-20
1 µM
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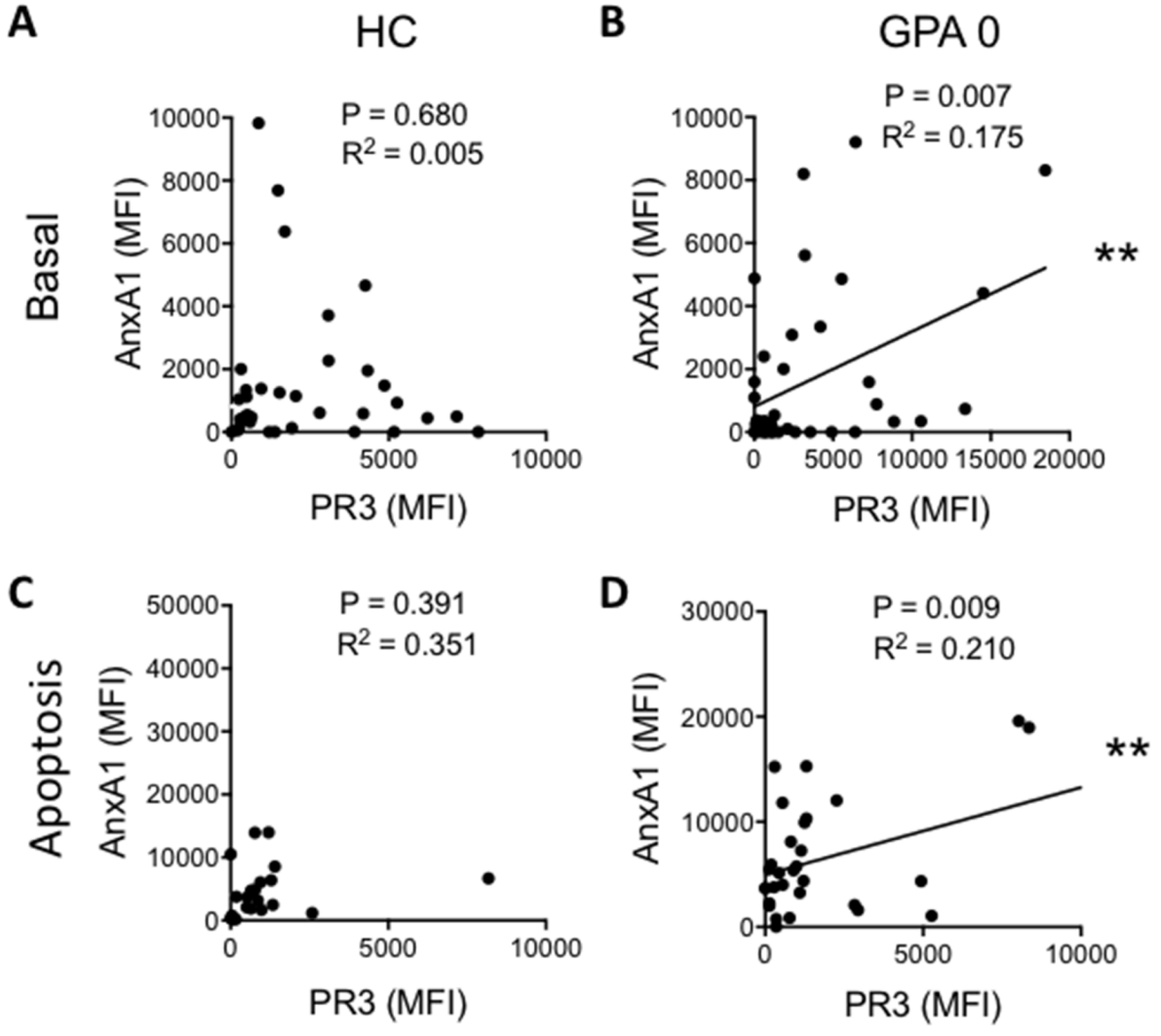
250 nM
125 nM
62.5 nM
-100
0
100
200
300
400
Time (s)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Basal
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PR3 (MFI)
C
D
Apoptosis
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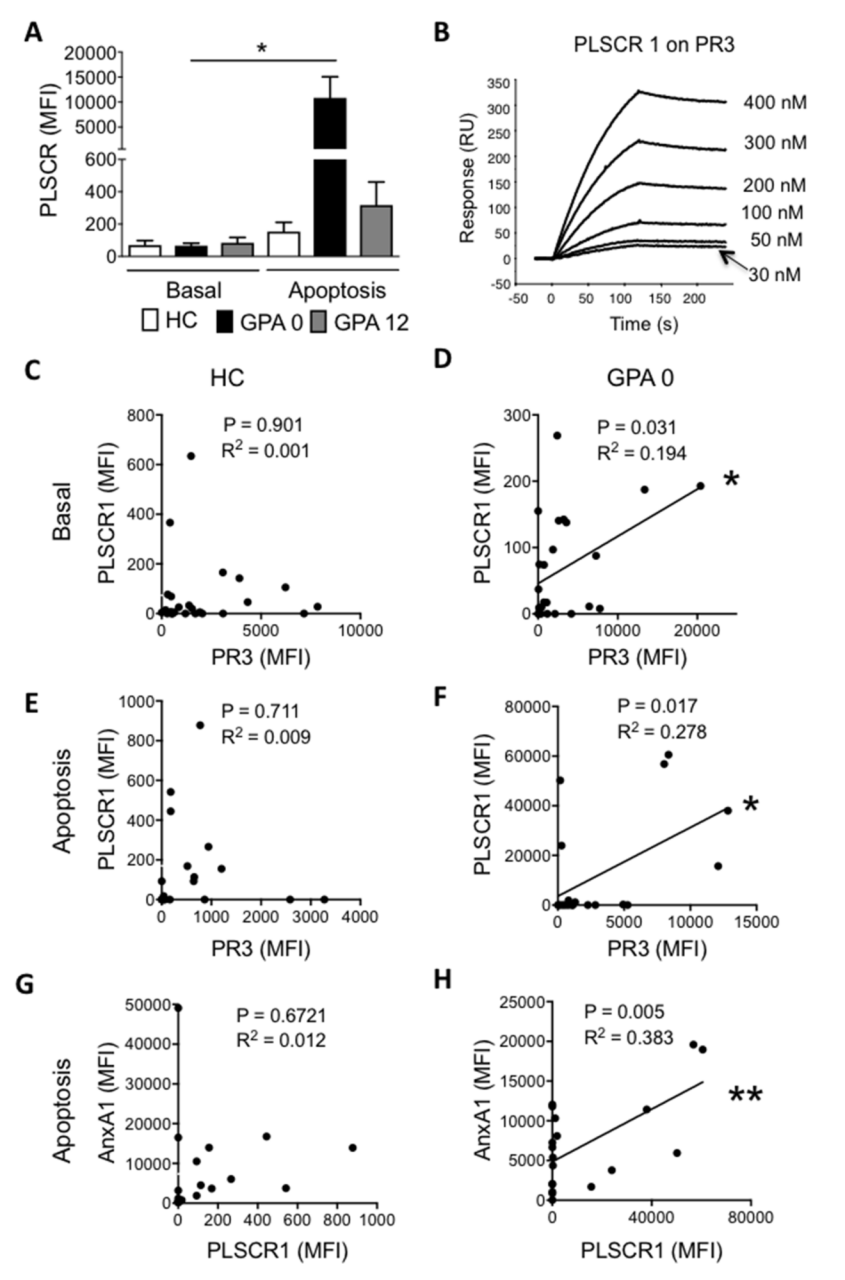
AnxA1 (MFI)
PR3 (MFI)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PLSCR (MFI)
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600
400
200
0
*
Basal
Apoptosis
HC
GPA 0
GPA 12
B
PLSCR 1 on PR3
Response (RU)
350
300
250
200
150
100
50
0
-50
-50 0 50 100 150 200
Time (s)
400 nM
300 nM
200 nM
100 nM
50 nM
30 nM
C
HC
Basal
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R2 = 0.001
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800
600
400
200
0
0 5000 10000
PR3 (MFI)
D
GPA 0
P = 0.031
R2 = 0.194
*
PLSCR1 (MFI)
300
200
100
0
0 10000 20000
PR3 (MFI)
E
Apoptosis
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R2 = 0.009
PLSCR1 (MFI)
1000
800
600
400
200
0
0 1000 2000 3000 4000
PR3 (MFI)
F
P = 0.017
R2 = 0.278
*
PLSCR1 (MFI)
80000
60000
40000
20000
0
0 5000 10000 15000
PR3 (MFI)
G
Apoptosis
P = 0.6721
R2 = 0.012
AnxA1 (MFI)
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40000
30000
20000
10000
0
0 200 400 600 800 1000
PLSCR1 (MFI)
H
P = 0.005
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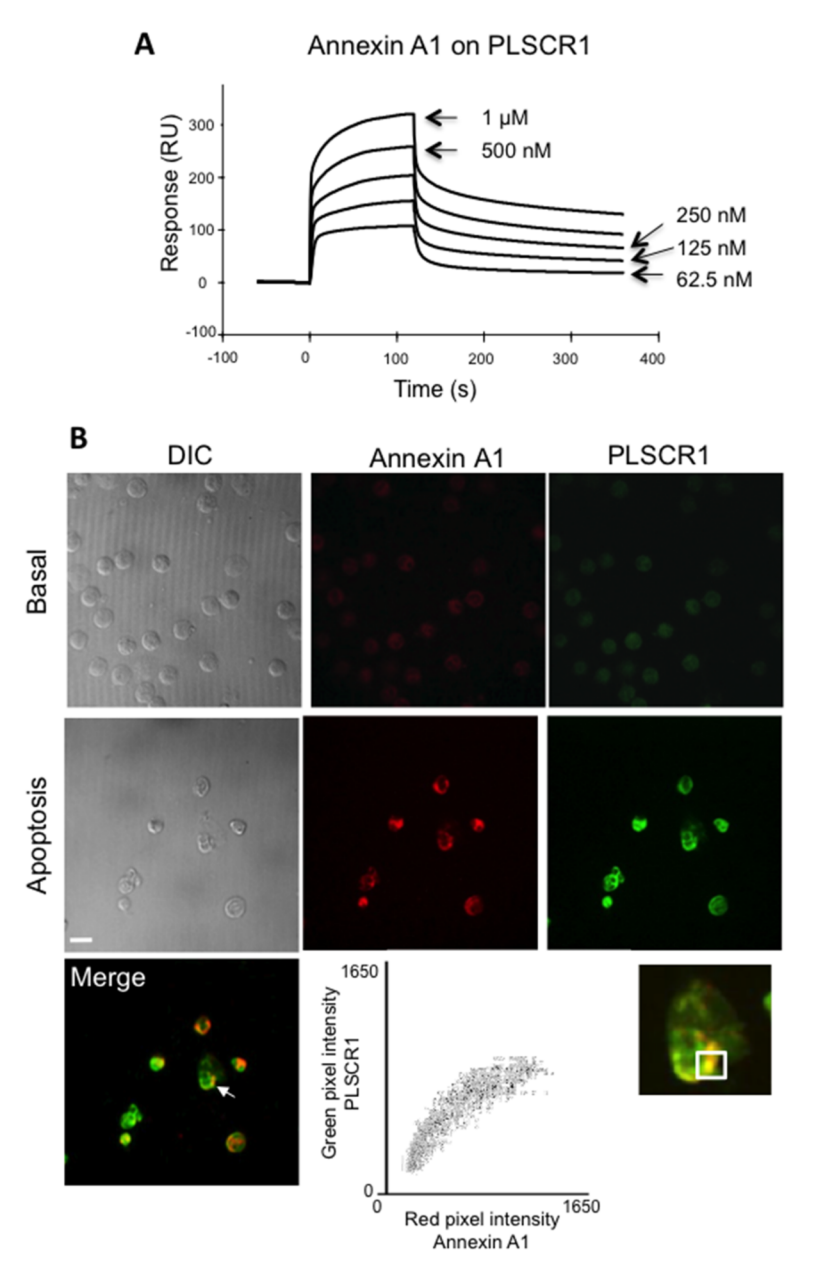
0 40000 80000
PLSCR1 (MFI)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250 nM
125 nM
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-100
0
100
200
300
400
Time (s)
B
DIC
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Apoptosis
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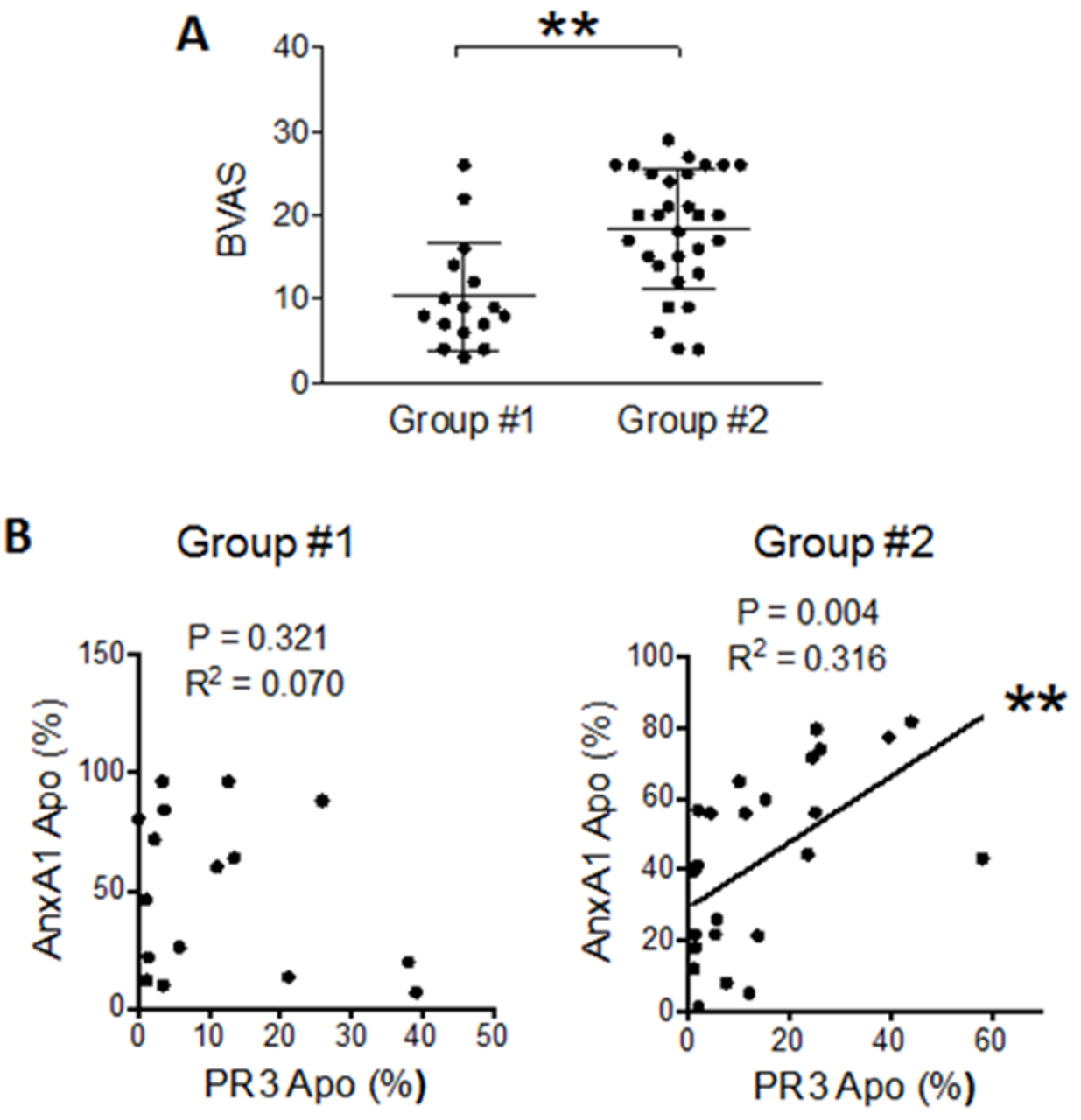
Red pixel intensity
Annexin A1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**
40
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20
10
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Group #1
Group #2
B
Group #1
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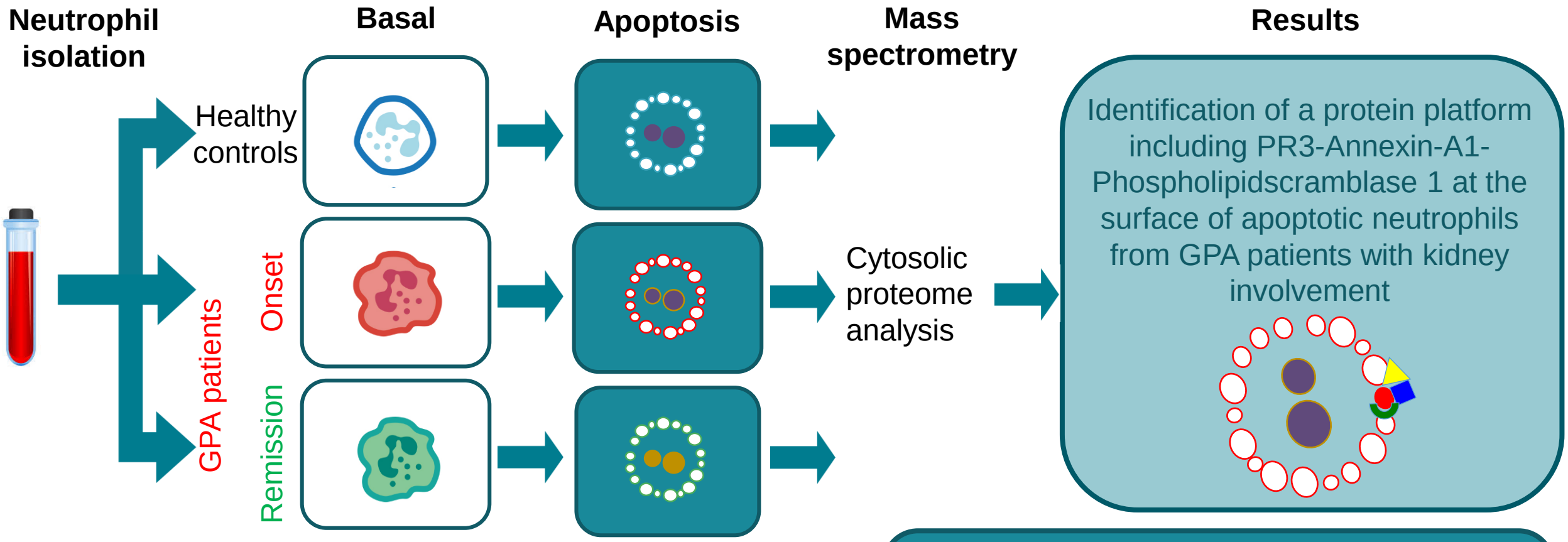
R2 = 0.070
150
100
50
0
AnxA1 Apo (%)
0
10
20
30
40
50
PR3 Apo (%)
Group #2
P = 0.004
R2 = 0.316
**
100
80
60
40
20
0
AnxA1 Apo (%)
0
20
40
60
PR3 Apo (%)

Proteomic analysis of neutrophils in ANCA-associated vasculitis reveals a dysregulation in proteinase 3-associated proteins such as annexin-A1 involved in apoptotic cell clearance.



GPA : granulomatosis with polyangiitis

CONCLUSION:

Membrane expression of PR3 partners could represent new markers of disease activity and therapeutic perspective in GPA

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