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Profiling of the Peripheral Blood Mononuclear Cells Proteome by Shotgun Proteomics Identifies Alterations of Immune System Components, Proteolytic Balance, Autophagy, and Mitochondrial Metabolism in Glaucoma Subjects

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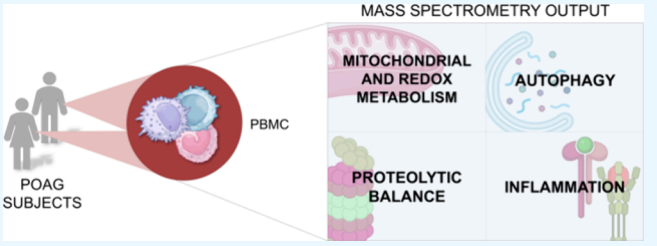
ABSTRACT: Glaucoma is a chronic optic neuropathy and is the second cause of irreversible blindness worldwide. Although the pathogenesis of the disease is not fully understood, the death of retinal ganglion cells and degeneration of the optic nerve are likely promoted by a combination of local and systemic factors. Growing attention has been paid to nonintraocular pressure risk factors, including mechanisms of inflammation and neuroinflammation. Phenotypical and molecular alterations of circulating immune cells, in particular, lymphocyte subsets, have been documented in murine models of glaucoma and in human subjects. Very recently, oxygen consumption rate and nicotinamide adenine dinucleotide levels of human peripheral blood mononuclear cells (PBMC) have been proposed as biomarkers of disease progression, thus suggesting that immune cells of glaucoma subjects present severe molecular and metabolic alterations. In this framework, this pilot study aimed to be the first to characterize global proteome perturbations of PBMC of patients with primary open-angle glaucoma (POAG) compared to nonglaucomatous controls (control) by shotgun proteomics. The approach identified >4,500 proteins and a total of 435 differentially expressed proteins between POAG and control subjects. Clustering and rationalization of proteomic data sets and immunodetection of selected proteins by Western blotting highlighted significant alterations of immune system compartments (i.e., complement factors, regulators of immune functions, and lymphocyte activation) and pathways serving key roles for immune system such as proteolysis (i.e., matrix metalloproteinases and their inhibitors), autophagy (i.e., beclin-1 and LC3B), cell proliferation (Bcl2), mitochondrial (i.e., sirtuin), and energetic/redox metabolism (i.e., NADK). Based on these findings, this proteomic study suggests that circulating immune cells suffer from heterogeneous alterations of central pathways involved in cell metabolism and homeostasis. Larger, properly designed studies are required to confirm specifically how immune cellular alterations may be involved in the pathogenesis of both neuroinflammation and glaucomatous disease.



1. INTRODUCTION

Glaucoma represents a group of neurodegenerative conditions characterized by the progressive degeneration of the optic nerve and the irreversible loss of retinal ganglion cells (RGCs) with consequent impairment of the visual field.[1] According to the World Health Organization, glaucoma is the second cause of irreversible blindness, accounting for 4.2 million (2.2%) of the 191 million visually impaired individuals on a global scale. Aging is an established risk factor for open-angle glaucoma (OAG), and as life expectancy is constantly increasing, the number of people affected by the disease is estimated to significantly increase to 112 million by 2040 (Tham 2014).[1]

Primary open-angle glaucoma (POAG), the most prevalent clinical form of OAG, is a multifactorial disease with a pathogenesis that has yet to be fully described. Although elevated intraocular pressure (IOP) has long been associated with POAG, its reduction has been shown to decrease but not eliminate the risk of POAG development or progression.[2−4]

A broad array of alterations encompassing the turnover and remodeling of the eye's anterior chamber tissues, regulation of systemic and local hemodynamic factors, mechanisms of

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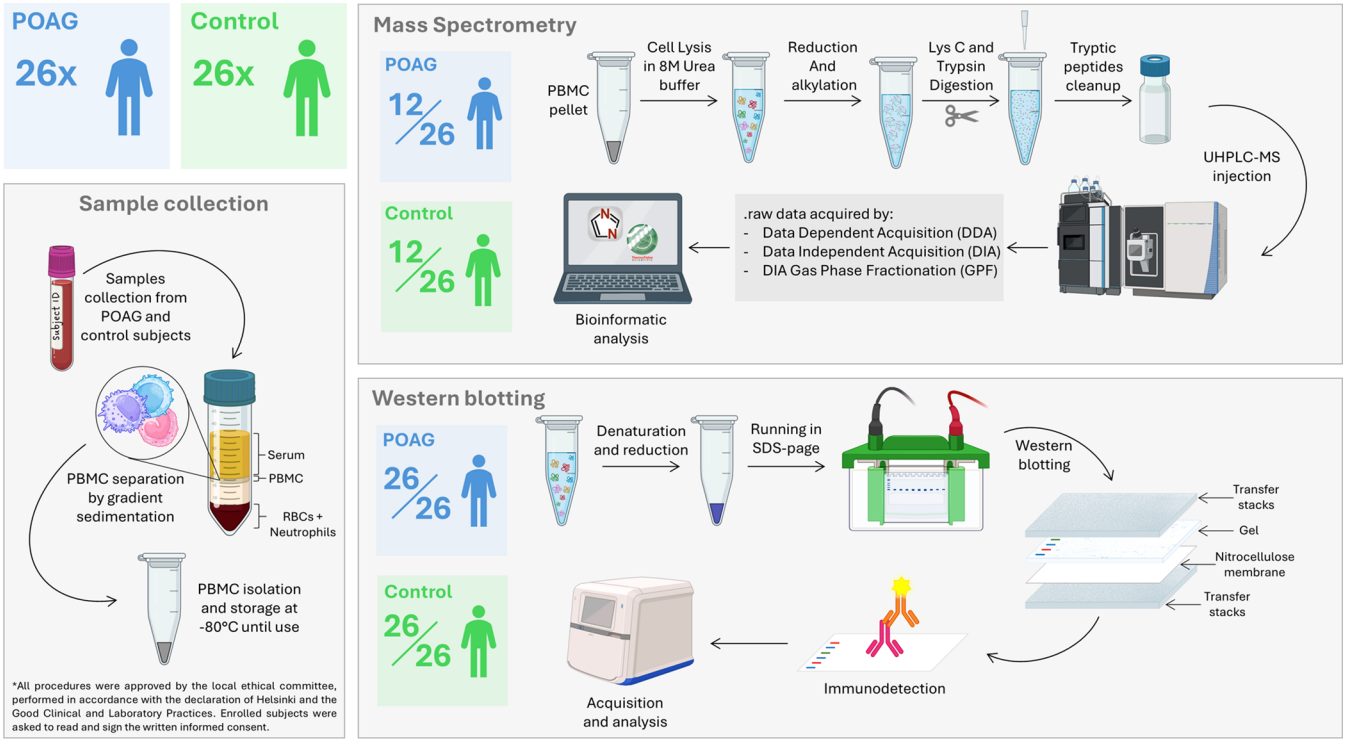

Figure 1. Schematic representation of the study design and experimental workflow.

inflammation, and genetic traits have all been suggested to combine and synergize during glaucomatous disease.[5−8] In this framework, great attention has been paid over the past decade to the pathological role of non-IOP factors in glaucoma etiology, including the process of neuroinflammation.

The paradigm of neuroinflammation in POAG and its relationship to hemodynamics and glaucomatous disease have been investigated in previous pilot work. Proteomics, metabolomics, and immune-enzymatic assays have been concordant in identifying increased levels of pro-inflammatory cytokines [e.g., interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), etc.], adhesion molecules (e.g., selectins), enzymes and their inhibitors [e.g., Matrix Metalloproteinases (MMPs), tissue inhibitor of matrix metalloproteinases (TIMPs)], stress-proteins [e.g., crystallins, heat shock proteins (HSPs)], and immune system components (e.g., complement) in local (e.g., aqueous humor) and systemic (e.g., plasma) fluids of glaucoma patients.[9−13] Unlike within other tissues, the dynamics of inflammation within the nervous tissues originates from two distinct cell lineages: (i) the resident glia (microglia and macroglia, represented by Muller cells and astrocytes in the retina), which provide support and protection to RGCs; and (ii) blood-borne immune cells. The cross-talk between these two lineages is thought to serve key roles in the homeostasis of nervous tissues and is currently being investigated also for brain neurodegenerative diseases including Alzheimer's and Parkinson's diseases.[14]

Research-based evidence has progressively highlighted that glial activation has prominent roles in shaping the adaptation of the retinal and optic nerve tissues to mechanical insults, which are characteristic of glaucoma pathogenesis (i.e., microbead injection into the anterior chamber of murine models). Furthermore, glial cells stimulate the adaptive and innate immunity during the early and advanced phases of disease pathogenesis.[15,16] More recently, preclinical data have pointed out phenotypical and molecular alterations of the immune system and in particular of T-lymphocytes primed against gut microbiota antigens, such as HSPs, during disease onset and progression.[17,18]

Very recently, oxygen consumption rate (OCR) and nicotinamide adenine dinucleotide (NAD) levels of peripheral blood mononuclear cells (PBMCs) have been found to highly correlate with disease progression, thereby emerging as biomarkers of progressive glaucoma.[19]

Therefore, the purpose of this pilot study was to shed further light on cellular and molecular pathways of PBMCs isolated from the peripheral blood of subjects diagnosed with POAG and nonglaucomatous controls (control) (Figure 1). To this aim, we interrogated global perturbations of proteome composition by high throughput mass spectrometry analyses run by label-free quantification (LFQ) adopting different acquisition modalities, including data-independent acquisition (DIA) using libraries generated in silico or refined by gas phase fractionation (GPF)-DIA, and further data-dependent acquisition (DDA).

This novel approach seeks to reveal differences between the proteome of POAG and control PBMCs, highlighting molecular signatures, including regulation of immune system compartments, proteolytic balance, mitochondrial, and redox metabolism, which are worth being explored further to elucidate their potential involvement in neuroinflammation and glaucomatous neurodegeneration.

2. RESULTS

2.1. Clinical Characteristics of Subjects Enrolled in the Proteomic Study. Twenty-five subjects (n = 26) for each experimental group were enrolled in the study (Figure 1). Mean age (±SD) was 71.81 (±11.49) for nonglaucomatous controls (control) and 71.42 (±7.06) for POAG subjects, and

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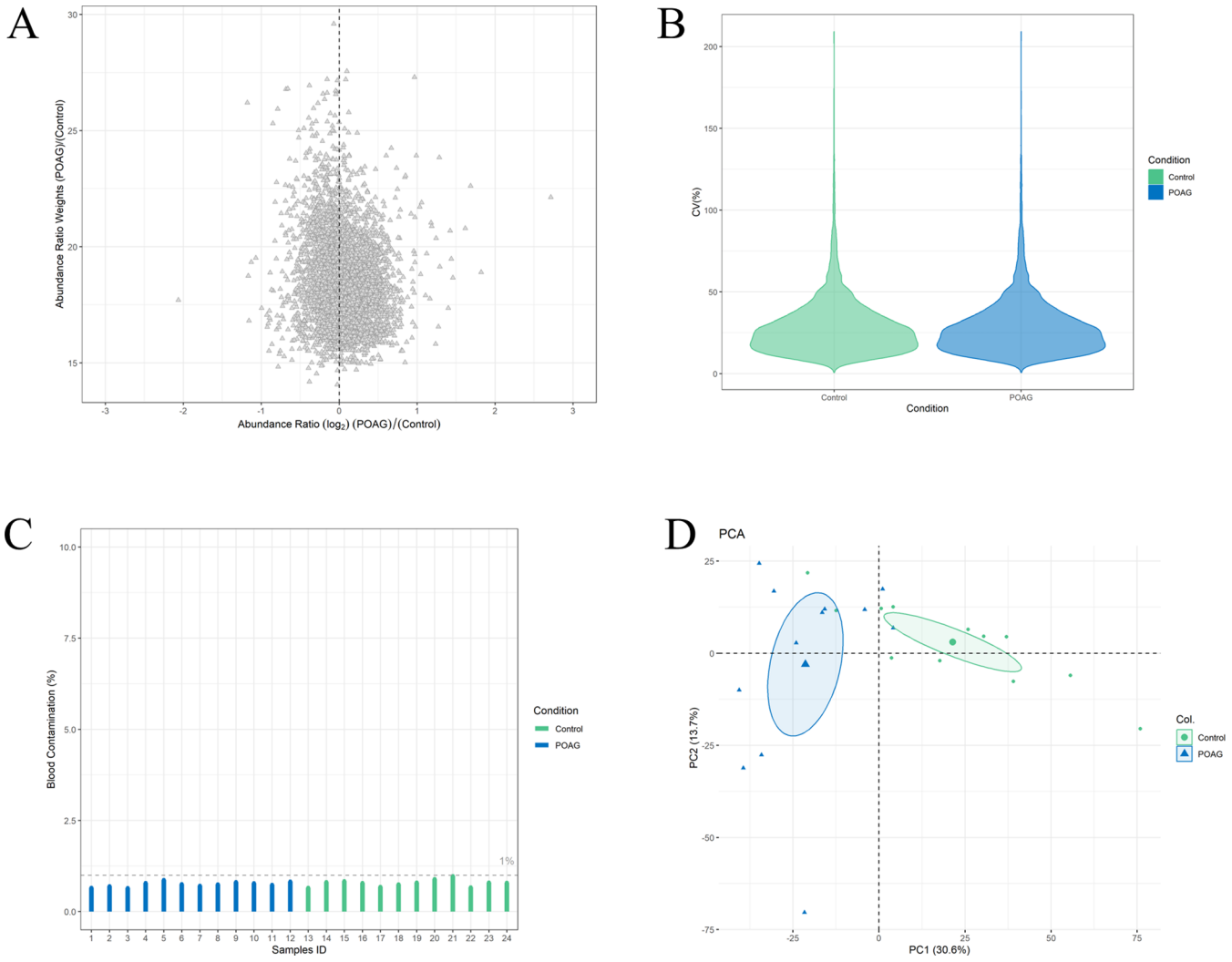

Figure 2. (A) Christmas tree plot: protein intensities were $\log_2$ transformed in the POAG/control ratio and plotted against the abundance weights, a parameter which identifies the $\log_2$ (intensities of POAG proteins + intensities of control proteins); (B) violin plots reporting the coefficient of variation (CV) of proteins identified in POAG and control PBMCs before any further normalization; (C) contamination of blood proteins in PBMC proteome was calculated; (D) principal component analysis (PCA) of POAG and control proteomes. The centroid for both experimental groups is indicated by a blue triangle (POAG) and a green dot (control).

regarding the gender ratio, it was 11:15 (males:females) for control and 13:13 for POAG (Table S1).

To minimize bias of interpretation, attention was paid to comorbidities as well as local and systemic medications.

The fraction of subjects with no known comorbidities was n = 8 for POAG and n = 9 for control. Hypertension was among the most prevalent comorbidities across all subjects enrolled (n = 16 for POAG, n = 13 for control). In these subjects, angiotensin-converting enzyme inhibitors (ACE) were the most represented class of drugs (n = 10 for POAG, n = 5 for control), followed by β-blockers (n = 5 for POAG, n = 8 for control) and angiotensin receptor blockers (ARB) (n = 2 for POAG, n = 3 for control). No subjects reported local or systemic drug use that would interfere with the study outcomes. Exclusions included severe comorbidities and the use of drugs with immunosuppressive activities.

To explore the heterogeneity of structural and functional damage across POAG subjects, we enrolled

(a) n = 4 fast progressor subjects, identified by a visual field rate of progression >−1 dB/y;
(b) n = 12 subjects with controlled glaucoma defect;
(c) n = 10 subjects without available visual field data including those who received their first glaucoma diagnosis the day of blood withdrawal.

Considering the eye with the worst visual field test, two subjects demonstrated better than MD −6 dB, 5 subjects were between −6 and −12 dB, and 9 had MD worse than −12 dB. Additional demographic and clinical details are shown in Table S1.

2.2. DIA Approach Identified >4000 Unambiguous Proteins in POAG and Nonglaucomatous Cataract PBMCs. A pilot shotgun proteomics study was undertaken enrolling the n = 12 subjects group (demographic and clinical parameters are introduced in Supporting Information 1) to compare the proteome of PBMCs of POAG and control groups. As described in the Materials and Methods section, spectra were acquired by three different MS modalities. The data analysis discussed below refers to the submission of DIA.raw files to DIA-NN software under library-free search, that is using the experiment-specific DIA library generated from the input files, in accordance with the software instructions (Demichev et al.[32]). However, the overall

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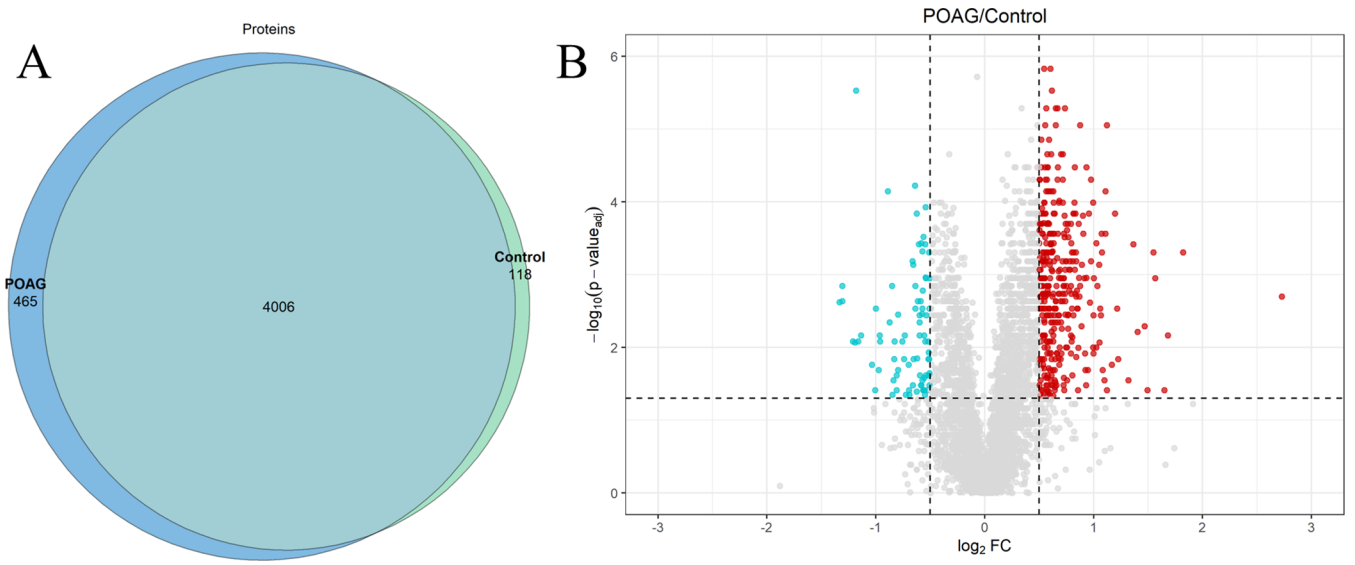

Figure 3. (A) Venn diagram showing the proteins identified in POAG and control experimental groups; (B) volcano plot showing the upregulated (red) and downregulated (turquoise) proteins in the POAG/control ratio. Dashed lines set the threshold used for labeling a protein as differentially represented. Filters were set as $0.5 \leq \log_2 FC \leq 0.5$ and $p \leq 0.05$ after Benjamini–Hochberg correction. This last value is graphed as $-\log_{10}(p\text{-value}_{adj})$ along the y axis. FC: fold change.

biological outcome was confirmed also by DDA (Tables S2 and S3 and Figures S1 and S2) and by an additional DIA analysis run with different technical parameters to enable the generation of a GPF-refined library (not discussed in detail) from the samples.

First, in the output file, proteins were filtered according to the following criteria: (a) q-value (Lib.Q.Value in the output matrix) ≤ 0.01; (b) identification of at least one proteotypic peptide; (c) identification of the protein in $\geq$9 out of 12 subjects of the same experimental group. Different visualization strategies were then used to analyze the technical soundness of the experiment. The Christmas tree plot (Figure 2A), which shows the $\log_2$-fold change (FC) ratio of proteins identified versus their abundance (i.e., weight), was consistent with a comparable distribution of data between POAG and control groups. The coefficients of variation (CVs) were <30% for the vast majority of all proteins identified and the shape of CV distribution overlapping between POAG and control (Figure 2B). Considering the phenotypical variability between subjects, this parameter was interpreted as promising for further interpretation of the data; the contamination with blood proteins was negligible (<1% of all proteins) for all samples enrolled (Figure 2C).

The principal component analysis (PCA) plot highlighted a clear separation of POAG and control groups across PC1 and PC2 (Figure 2D).

Even adopting stringent criteria for protein identification, the approach identified a remarkable 4006 proteins common to POAG and control groups. Additional 465 proteins were identified as POAG-specific and 118 as control-specific (Figure 3A).

Based on the filter posed and considering that DIA approaches are less burdened by the missing values problem as DDA actually does, the proteins identified as group-specific were not filtered out. The identity of these proteins is reported in Tables S2 and S3 for POAG and control, respectively.

Although the distribution of data was clearly comparable between experimental groups, a class-specific quantile normalization strategy, which was found to outperform the canonical quantile normalization strategy, was applied to normalize the intensity values before analyzing for differentially expressed proteins (DEPs).[20] The intensities ratio of proteins common to POAG and control groups were calculated by setting a $\log_2 FC \geq 0.5$ and ≤ -0.5 (expressed as POAG/control ratio). The statistical significance of observations was determined by the Mann–Whitney nonparametric test ($p \leq 0.05$) and p values corrected by the Benjamini–Hochberg test for multiple hypotheses (Figure 3B).

Interestingly, a significant proportion of proteins (353) were found as upregulated in POAG PBMCs. Conversely, a lower number (82) of targets were identified as downregulated in the same experimental group. The full list of proteins upregulated and downregulated in the POAG/control ratio is provided in Tables S2 and S3, respectively.

2.3. Data Clustering and Rationalization (KEGG and GO) Highlights Alterations of Key Metabolic Pathways in POAG PBMCs. To reveal the terms enriched in POAG PBMCs, upregulated and exclusive proteins were subsequently analyzed by Gene Ontology (GO) and KEGG pathway. By inspecting the molecular function (MF) and biological processes (BP) charts (in both cases $p \leq 0.05$, plots show only terms identified with >10 and >15 proteins, respectively), Our results show POAG PBMCs were significantly enriched (proteins are identified by UniProt primary accession number) (Figure 4A,B, Figure S2, and Table S2):

1. Structural component of chromatin and chromatin and nucleosomal DNA binding proteins, as well as RNA polymerase II activity: histone deacetylase complex subunit SAP18 (O00422), DNA fragmentation factor subunit alpha (O00273), ATP-dependent RNA helicase DHX15 (O43143), pre-mRNA-processing factor 6 (O94906), U1 and U2 small nuclear ribonucleoproteins (P08579 and P08621), histone H1.3, H1.4, H3.3, and H4 (P16402, P10412, P84243, and P62805), DNA topoisomerase 1 (P11387); DNA-directed RNA polymerase II subunit RPB1 and RBP11a (P24928, P52435),

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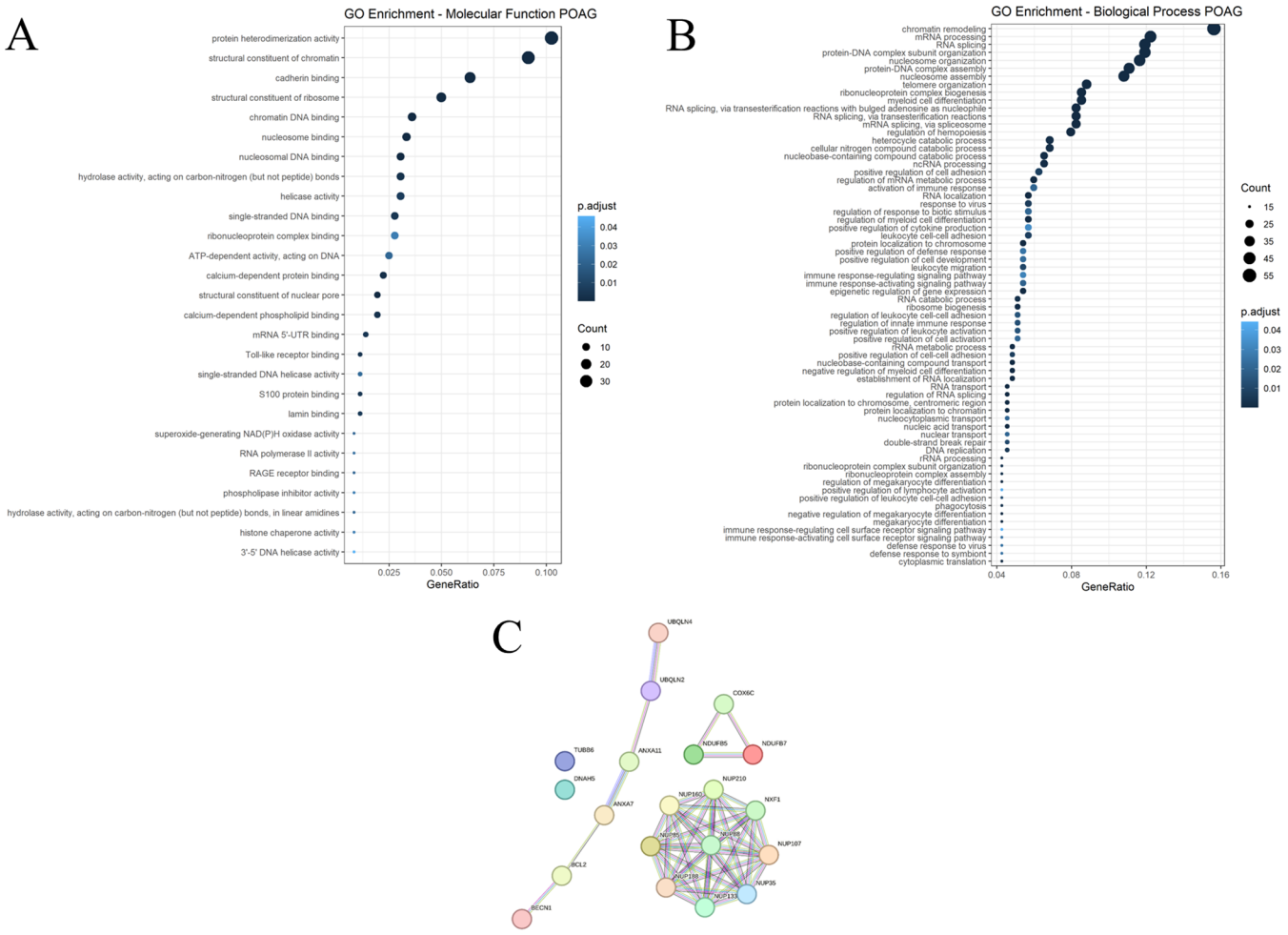

Figure 4. (A) Molecular function (MF) and (B) biological processes (BP) terms found enriched by submitting proteins upregulated in POAG PBMCs to GO. GeneRatio was calculated and data filtered for $p \leq 0.05$; (C) nodes and edges found as enriched by the KEGG pathway ($p \leq 0.05$).

DNA-directed RNA polymerases I, II, and III subunit RPABC3 (P52434), DNA topoisomerase 1 (P11387); histone deacetylase 2 (Q92769);

2. Hydrolase activity: neutrophil elastase (P08246), cathepsin G (P08311), myeloperoxidase (P05164), eosinophil cationic protein (P12724), granzyme M (P51124), MMP-9 (P14780);
3. Superoxide-generating NAD(P)H oxidase activity: NAD kinase (O95544), nicotinamide phosphoribosyltransferase (P43490), NADH dehydrogenase 1 beta subcomplex subunit 5 (O43674), DNAJ homologue subfamily C member 17 (H0YLV4), cytochrome c oxidase, subunit 6 (P09669), NAD-dependent protein deacetylase sirtuin-3, mitochondrial (Q9NTG7); aldehyde dehydrogenase (P05091);
4. Autophagy and apoptosis proteins: beclin-1 (Q14457) and Bcl2 (P10415); Bcl2 associated transcription factor 1 (Q9NYF8), apoptosis inhibitor 5 (Q9BZZ5);
5. A heterogeneous panel of proteins involved in immune system regulation: protein S100-A9 and S100-A12 (P06702, P80511), annexin A2 (P07355), A4 (P09525), A6 (P08133), A7 (P20073), A11 (P50995), brain acid soluble protein 1 (P80723), dynein axonemal heavy chain 5 (Q8TE73);
6. Toll-like and RAGE receptor binding: rab11 family interacting protein 1 (Q6WKZ4), myeloid differentiation primary response protein Myd88 (Q99836), ras-related protein Rab-3D (O95716)), TRAF3-interacting JNK-activating modulator (Q9Y228), stimulator of interferon genes protein (Q86WV6), and inactive ubiquitin thioesterase OTULIN (Q9NUU6).

In addition, enrichment of proteins critical for lineage determination and controls of proliferation of lymphoid system and of T-cell immunity were documented: DNA-binding protein Ikaros (Q13422) and Ets-related transcription factor Elf-1 (P32519), CD2 antigen (O95400), T-cell surface glycoprotein CD5 (P06127), and tyrosine-protein kinase ZAP-70 (P43403).

To further investigate the data set, upregulated proteins in POAG PBMCs were submitted to STRING Network software.[21] A meaningful 2428 edges for 348 nodes, with an average node degree of 14 and an average clustering coefficient of 0.465 ($p < 10^{-16}$) was documented, indicating that this protein data set had significant biological connections (Figure S3).

Regarding the KEGG analysis, a significant enrichment of different pathways ($p \leq 0.05$) (Figure 4C), namely, protein export (hsa03060), DNA replication (hsa03030), spliceosome (hsa03040), mRNA surveillance pathway (hsa03015), ribosome (hsa03010), RNA transport (hsa03013), Systemic lupus erythematosus (hsa05322), phagosome (hsa04145), and

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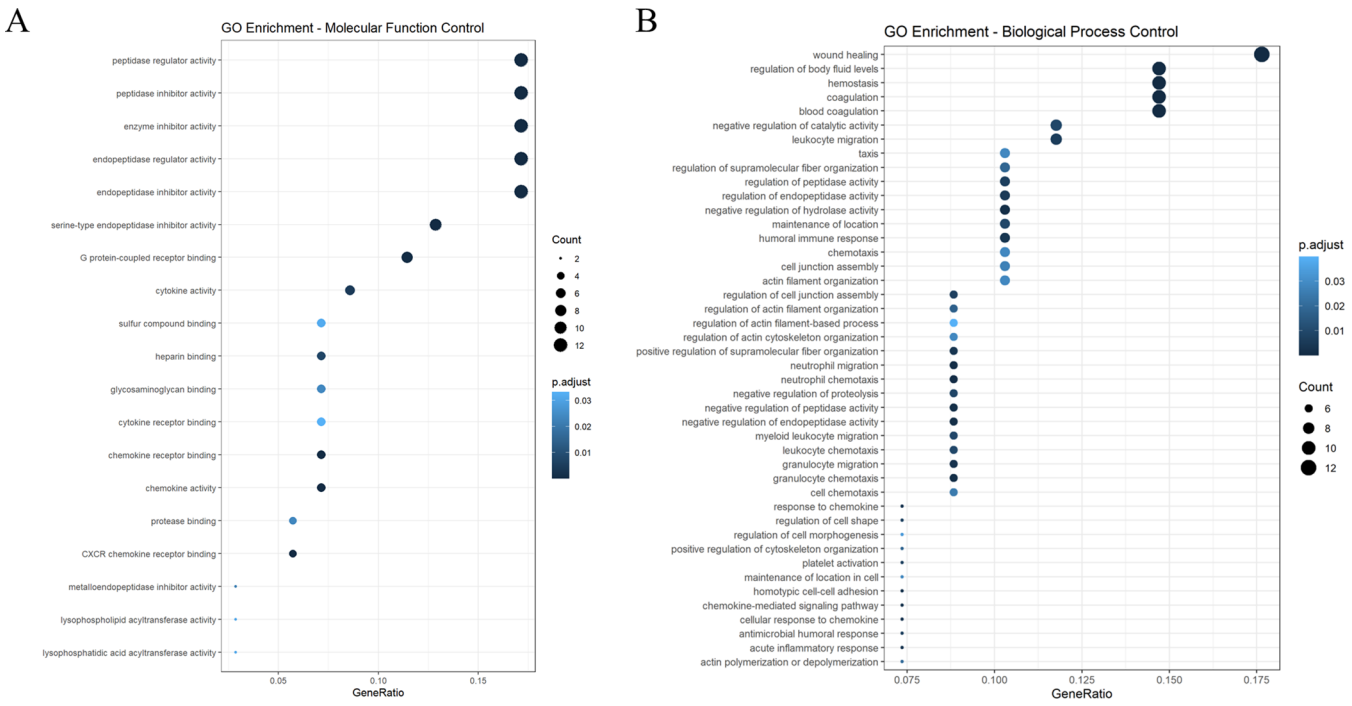

Figure 5. (A) Molecular function (MF) and (B) biological processes (BP) terms found to be enriched by submitting proteins downregulated in POAG (labeled as enriched in control PBMCs). GeneRatio was calculated and data filtered for $p \leq 0.05$. GO term "adaptive response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains" is replaced by "somatic hypermutations" to optimize image resolution.

amyotrophic lateral sclerosis (hsa05014), was documented in POAG PBMCs.

Interestingly, among nodes supporting this enrichment, there were proteins, such as beclin-1, Bcl2, ubiquilin-2 and ubiquilin-4, cytochrome c oxidase subunit, and NADH dehydrogenases that are robustly involved in pathological pathways of neurodegeneration, cell proliferation and death, and energy and redox metabolism (Figure 4C).

Based on these data, GO enrichment charts were further generated for proteins identified as downregulated in POAG PBMCs. For the sake of readership, these proteins are now labeled in figures as enriched in controls PBMC ($p \leq 0.05$) (Figure 5A,B, Table S3).

Of specific note, in this case, proteins specular to those identified as upregulated in POAG PBMCs were documented, namely:

1. Mechanisms of regulation of peptidase and protease activity: heparin cofactor 2 (P05546), TIMP-1 (P01033), TIMP-3 (P35625), alpha-1-antitrypsin (P01009), alpha-1-acid glycoprotein (P02763), and glia-derived nexin (serpin E2, P07093);
2. Soluble mediators of inflammation: C−C motif chemokine 5 (P13501), C-X-C motif Chemokine 5 (P42830), platelet basic protein (P02775), and platelet factor 4 (P02776);
3. Proteins involved in regulation of mitochondrial and autophagic processes through lipid mobilization: caveolae-associated protein 2 (O95810) autophagy-related protein 9A (Q7Z3C6), and atlastin-2 (Q8NHH9);
4. Complement factors and adhesion molecules: complement C3 (P01024), complement component C8 beta chain (P07358), intercellular adhesion molecule 2 (P13598), and c-type lectin domain family 1 member B (Q9P126).

2.4. Western Blotting Analysis of Selected Proteins Confirms Proteomic Data Sets. To strengthen the evidence regarding the dysregulation of pathways central for immune system function, including those identified by the KEGG analysis and previously mentioned (Figure 4C), a selection of proteins was interrogated by Western blotting (Figure 6):

(a) Beclin-1 together with microtubule-associated light chain I and II (LC3B−I, LC3B−II), which are validated markers of autophagy;
(b) TIMP-1, TIMP-3 and MMP-9;
(c) Bcl2;
(d) NAD kinase (NADK).

To strengthen the outcome, the intensity of beclin-1, Bcl2, and NADK calculated during the first analysis on $n = 12$ subjects was used to impute the number of additional subjects to enroll to reach a power of >90% (considering $\alpha = 0.05$). This threshold was chosen to partially compensate for the semiquantitative nature of the study's approach.

The analysis indicated that enrollment of 20 or more subjects for each experimental group would provide a power of >90 for NADK, Bcl2, and beclin-1. Therefore, $n = 14$ additional subjects for each experimental condition ($n = 26$ subjects per group in total) were enrolled.

In our analysis, Wb data indicated that TIMP-1 levels were slightly higher in the control subjects, although the observation did not reach statistical significance. Conversely, TIMP-3 was detectable, at least for the sensibility of the approach, exclusively in control subjects. Interestingly, a specular behavior was documented for MMP-9, which is targeted by TIMP-3 in vivo, and was detected almost exclusively in POAG subjects.

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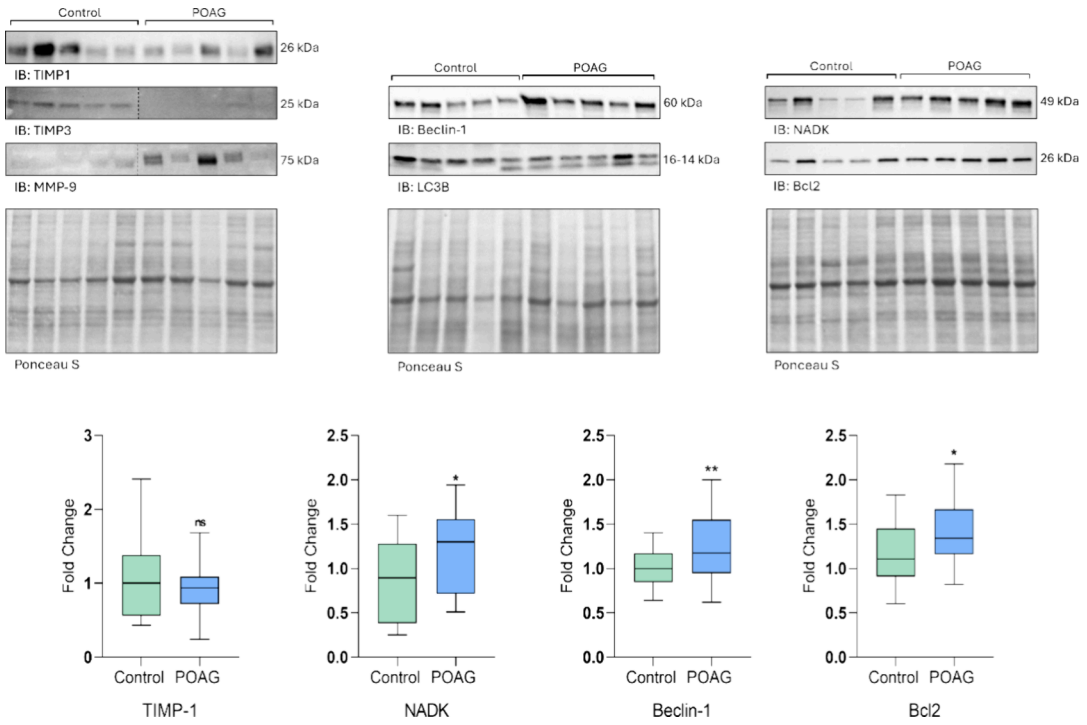

Figure 6. Western blotting panel analysis of selected proteins of $n = 26$ POAG and $n = 26$ control subjects. A representative blot is shown. Dashed lines in the TIMP-3 and MMP-9 blots indicate that the original image was cropped. Uncropped images are provided in the Figure S4. Total proteins as stained by Ponceau S were used for normalization (see the Materials and Methods for further details). The box plots show the median and min max values of protein intensities as fold change with respect to nonglaucomatous control subjects. Unpaired τ test was used for statistical analysis after having verified a normal distribution of data (Shapiro−Wilk test); $*p \leq 0.05$; $**p \leq 0.01$. The box plot and statistical analysis are not provided for TIMP-3, MMP-9, and LC3B as the proteins turned out to be below the detection limit for one of the two experimental groups. Regarding LC3B, note that the proteins migrate as a doublet band (16−14 kDa). LC3B−II (14 kDa), which corresponds to LC3B−I (16 kDa) conjugated with phosphatidyl ethanolamine (PE), migrates faster due to its higher hydrophobicity. Lipidation of LC3B−I occurs during autophagy activation.

Regarding autophagy, beclin-1 levels were significantly upregulated in POAG subjects. To validate this finding further, filters were probed with an anti-LC3B antibody, although the protein was not detected by the MS approach. LC3B−II levels, which more truthfully represent the rate of pathway activation, were above the detection limit in the case of control PBMCs, and they were robustly detected in POAG cells. Furthermore, compared to control PBMCs, the intracellular content of Bcl2 was significantly increased in POAG cells, as well as that of NADK, a cytosolic enzyme involved in the generation of $NADP^+$ using NAD^+ as precursor.

Thereafter, to verify the effect of potential confounders, the relationship between beclin-1, Bcl2, and NADK intensities with the main ocular, demographic, and systemic parameters of the study subjects was computed by multivariate regression analysis in POAG and control groups (Figure 7A,B). We included in the model covariates with observed values in ≥15% subjects.

The analysis did not retrieve any significant relationship ($p \leq 0.05$) between individual protein intensities and main ocular and systemic parameters, nor for control or POAG groups, with the exception of Bcl2 and treatment with oral acetazolamide ($p \leq 0.047$). However, the assumption of oral acetazolamide was documented for 4 out of 26 subjects and was not reported in control subjects.

3. DISCUSSION

To the best of our knowledge, this pilot study is among the first to specifically characterize the proteome of circulating PBMCs isolated from POAG and nonglaucomatous control subjects (control). By coupling DIA and DDA approaches with Western blotting studies, we identified dysregulation of relevant pathways for immune cell homeostasis in POAG PBMCs. Specifically, the analysis highlighted differences in proteins and GO terms related to mechanisms of immune system compartments, transcription and translation processes, extracellular proteolysis, autophagy, and mitochondrial and redox metabolism.

Over the last two decades, independent preclinical studies have suggested that dysregulation of immune system compartments plays a role in the pathogenesis of glaucoma and optic nerve degeneration, with several data showing phenotypical and molecular alterations of circulating immune cells.[7,9,11,16,18,22−28] Murine models and in vitro assays have specifically highlighted that redox unbalance increases the rate of T-cell activation by retinal glial cells and that T-lymphocytes primed against HSP proteins trigger aberrant rates of RGC and optic nerve degeneration.[26,27] Further studies confirmed the detection of increased plasma titers of autoantibodies targeting stress proteins, such as αB-Crystallin, HSP27, HSP60, and retinal deposits of immunoglobulin G (IgGs).[9,22,25] Although the autoimmune hypothesis has not gained definitive validation, a deep involvement of T-cell subsets in glaucoma pathogenesis has been strengthened by more recent research

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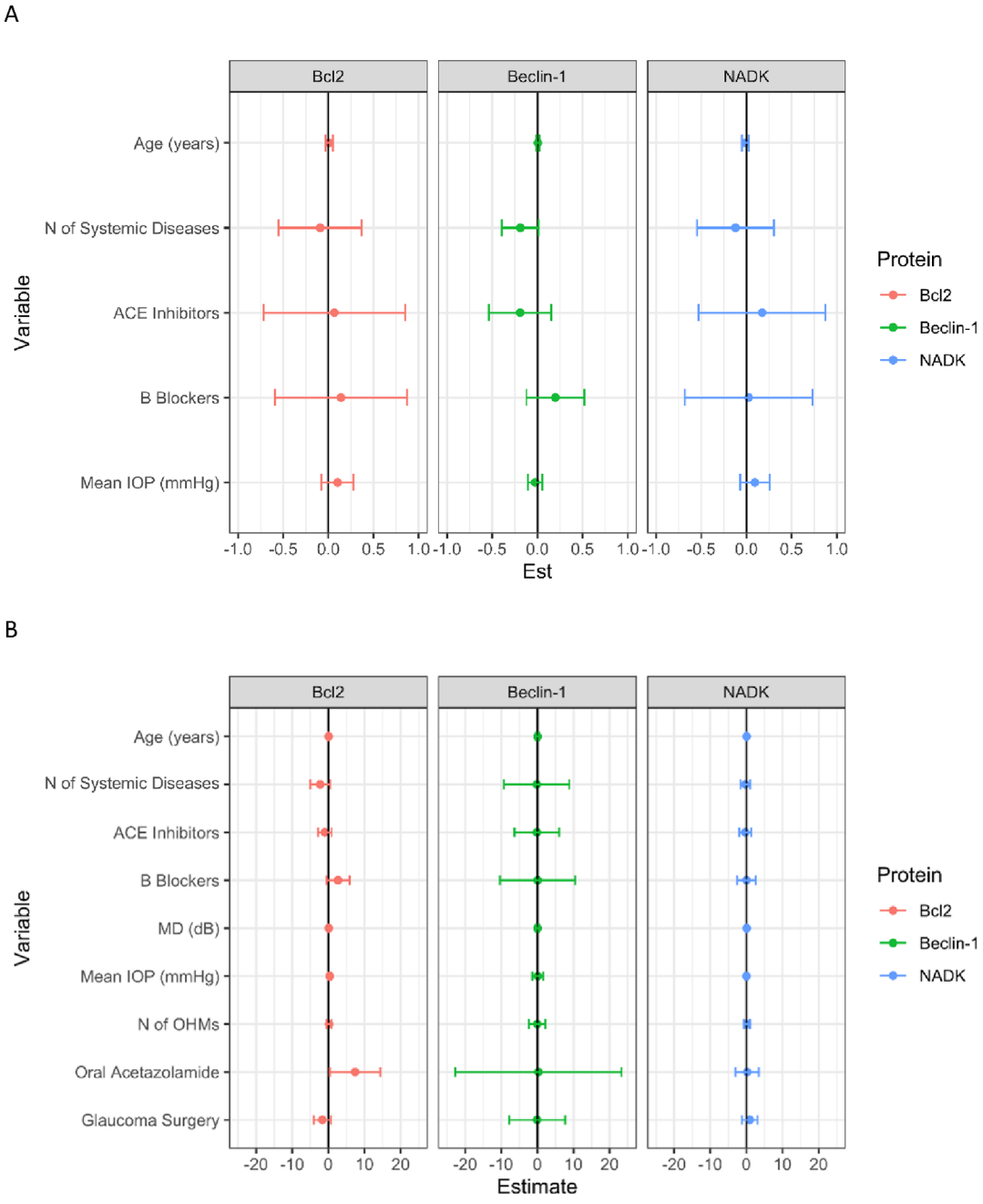

Figure 7. Plots showing the relationship between intensities of Bcl2, beclin-1 and NADK determined by Western blotting analysis and main ocular, demographic, and systemic parameters for control (A) and POAG (B) subjects, namely, age, number (*N*) of systemic diseases, therapy with ACE inhibitors, β (B)-blockers or oral acetazolamide, mean deviation (dB) of visual field loss, number (*N*) of ocular hypotensive medications (OHMs), mean IOP (mmHg), glaucoma surgery. The intervals of confidence are reported.

on human subjects and murine models. Specifically, two different studies reported altered frequencies of regulatory T-cells in glaucoma subjects.[23,28] A significantly higher percentage of $CD4^+CD25^+$ T-cells, which exert immunosuppressive functions by inhibiting the release of interleukin-2, was observed in glaucoma subjects with respect to healthy controls.[23] In another study, a significantly lower frequency of $CD4^+$ (or $CD8^+$)/$CD25^+$/$FoxP3^+$ T-regs within the entire $CD4^+$ (or $CD8^+$) population, despite similar frequencies of $CD4^+$ or $CD8^+$ T-cells, or Th1, Th2, or Th17 subsets, was again detected in glaucoma subjects.[28]

It is worth considering that these populations serve key roles for the crosstalk between immune system compartments and resident glia of nervous tissues and, further, promote the proteostasis balance through enhanced clearance of proteotoxic aggregates.[29−31] Additional molecular clues regarding the role of adaptive immunity in glaucoma onset and progression have recently come from murine models in which an acute IOP elevation by microbead injection was used. $Rag1^{-/-}$ mice, which are depleted of T- and B-cell subsets, were observed to retain significantly higher numbers of surviving RGC when compared to immune-competent control mice after 16 weeks of elevated IOP.[17] In another experimental mouse model, $\beta7^+$ $CD4^+$ T-cells were documented to home back to the gut during the acute phase of glaucoma, where they underwent transcriptional reprogramming and upregulation of pathways

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typically enriched in autoimmune diseases, bacteria responses, mucosal immunity, and glial activity.[18] This process was documented as essential for progressive RGC damage in diseased mice.[18] Remarkably, in this study, $CD4^+$ T-cells expressing a gut-homing integrin $\beta7$ were found to show increased frequency in glaucoma subjects and to correlate with the disease stage.

The preponderance of preclinical data, together with mounting scientific interest in the role of the immune system in neurodegeneration, necessitates further investigation.

In this framework, profiling of the proteome of PBMCs isolated from POAG and control subjects looks relevant to obtain a comprehensive view of altered pathways that, if confirmed by future studies, may underscore immune trajectories and immune-mediated glaucoma damage, further offering new perspectives for the identification and validation of novel diagnostic or follow-up biomarkers. In doing so, it is important to mitigate potential biases including age, the presence of systemic severe comorbidities, and the use of drugs with immunosuppressive activities. In our analysis, we excluded these criteria to reduce error, with hypertension being the most common comorbidity documented, and its frequency was balanced between the two experimental groups. Medications used to treat hypertension were also the most commonly reported in both study groups. Our analysis also included heterogeneity of functional and structural damage across enrolled individuals to rule out the influence of clinical status.

To maximize the technical soundness of data, all samples were subsequently digested and analyzed concurrently by shotgun/bottom-up LFQ proteomic strategies and spectra acquired by three different modalities, namely, DIA, DIA-GPF, and DDA. DIA approaches are gaining growing relevance in mass spectrometry studies as they boost the identification of peptides/proteins and are less burdened by the missing value problem.[32−35] Regardless of technical design, it is worth pointing out that the general experimental outcome was very comparable between the approaches used, and quality controls provided a solid basis for further interpreting the results.

Data clustering and rationalization highlighted robust differences in several biological processes and molecular functions. First, it is worth pointing out that a global intense metabolism of POAG PBMCs may be speculated on the basis of the very marked enrichment in chromatin remodeling, DNA/protein interactions, and transcriptional and translational activities documented in diseased subjects.

Although the proteins identified do not allow one to draw specific conclusions, data clustering and rationalization cast light on different mechanisms which may support the intense metabolic program POAG PBMCs experience.

Control PBMCs showed significant enrichment of complement immunity. Several complement factors (C3 and C8) were effectively enriched in control (or down-regulated in POAG PBMCs, indeed). Nevertheless, complement cascade was proposed to serve key roles in neuroinflammation and RGC death in glaucoma experimental models.[36,37]

Conversely, POAG PBMCs showed significant enrichment to mechanisms of immune system activation and signaling cascades. Among the several proteins identified, we found Elf1, Ikaros, ZAP-70, and CD5 significantly upregulated in POAG PBMCs in proteomic data sets. Although these proteins were reported to serve prominent roles for T-cells, activities for B-cell functions and differentiation were further reported.[38−44] Hence, additional studies are demanded to address the biological rationale of their upregulation, whether one or more specific cell types among those included in the PBMCs population carry this alteration, and to confirm whether POAG PBMCs have a prominent T-cell polarization.

By examining data through a deeper molecular and metabolic perspective, data clustering and rationalization retrieved the dysregulation of a panel of specific pathways deeply involved in immune system regulation, inflammatory polarization, and signaling cascades. In this regard, POAG PBMCs were highly enriched in proteins involved in inflammatory Ca^{2+}-dependent signaling cascades in lymphocytes, such as annexin A4, soluble brain acidic protein 1, S100A9, and S100A12.[45,46]

These findings may help elucidate the dynamics of pro-inflammatory polarization of infiltrating immune cells, with a particular focus on nuclear factor kappa B (NF-kB) signaling cascade and the cross-talk of these cells with resident glia in orienting optic nerve damage and RGCs death and survival. Annexin A4, in particular, was linked to plasma membrane repair processes and biomechanical strains associated with glaucoma pathogenesis, through mechanisms involving annexin extracellular aggregation, membrane and cytoskeleton dynamics mediated by F-actin.[45,47]

A relevant observation concerns the mechanisms of extracellular and intracellular proteolysis and proteostasis. Proteomics and Western blotting analyses were concordant in identifying a drop of several natural inhibitors of peptidases and proteases (e.g., $\alpha1$-antitrypsin, TIMP-3, etc.) mirrored by an increase of proteolytic enzymes, such as MMP9 in POAG vs control PBMCs. In this regard, MMP9 is the most important inducible and pro-inflammatory MMP and is naturally inhibited by TIMP-3.[48] Nevertheless, deficiency in MMP9-deficiency was shown to be protective for RGC survival after optic nerve ligation in transgenic mice, while increased MMP9 levels in eye fluids were reported to represent a risk factor for POAG development and optic nerve degeneration.[49,50] Since a relevant source of MMP9 is represented by immune cells, infiltration of eye tissues by MMP9-enriched and TIMP3-deficient PBMCs may have implications for local tissue remodeling and damage.

In addition to proteolysis, our attention was then paid to proteins and pathways, such as autophagy, control of cell proliferation, and mitochondrial metabolism, that act synergistically in the regulation of the immune system.[51−53]

Autophagy, besides serving key roles for optic nerve degeneration processes, is known to bridge innate and adaptive immune responses supporting effector processes, antigen presentation, lymphocyte homeostasis, regulation of cytokine production, and cross-talk between immune cells.[51] In this study, beclin-1, which is a prominent autophagy marker, was significantly upregulated in POAG PBMCs in both proteomics and Wb data sets, and such an increase was not associated with confounding clinical and demographic factors. This finding suggested the importance of measuring the level of another recognized marker of autophagy function, LC3B−II, by Wb. Although data must be interpreted with caution, since a lysosomal inhibitor was not delivered, LC3B−II was below the detection limit in the case of control PBMCs, and robustly detected in POAG cells.[54]

In POAG PBMCs, we found a concomitant upregulation of Bcl2 (an antiapoptotic factor and a known interactor of beclin-1), with a downregulation of Atg9A and atlastin-2, which are involved in essential lipid trafficking processes for autophagy

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and mitochondria. It is important to note that a possible association with oral acetazolamide intake was observed with the upregulation of Bcl2, although the drug was taken by a very limited fraction of subjects (4 of 26). Taken together, these results demand careful evaluation to further elucidate the precise mechanisms of cell proliferation and survival and the functional status of these cells.[55,56]

Interestingly, in this framework, the proteomic data sets and Wb were further concordant in identifying upregulated levels of NAD kinase (NADK), without any associations with confounding factors. This enzyme, which converts NAD^+ to $NADP^+$, serves roles for energy metabolism and for the generation of metabolic precursors of specific pathways, including the pentose phosphate pathway.[57,58]

In this regard, retinal NAD^+ levels progressively decrease during aging, rendering neuronal cells more vulnerable to different insults. In a pioneering study, oral administration of vitamin B_3 (precursor of NAD^+ metabolite), and/or gene therapy (i.e., *Nmnat1,* which is an NAD^+-producing enzyme, gene therapy) were highly protective either for preventing disease onset and for therapy in glaucoma-prone mice.[59]

Very recently, the oxygen consumption rate (OCR) of PBMCs was associated with faster visual field progression in glaucoma patients treated by lowering IOP and NAD levels were found to be highly associated with the OCR.

Therefore, for the first time, OCR and NAD levels were proposed as biomarkers of progressive glaucoma.[19]

Nevertheless, the safety and efficacy of NAD supplementation in delaying glaucoma progression is currently investigated by a phase III clinical trial (ClinicalTrials.gov Identified: NCT05405868).

In our study, in addition to NADK, which is mainly cytosolic, several additional mitochondrial enzymes involved in redox metabolism and mitochondrial respiration were found to be upregulated in POAG PBMCs. Among them, it is worth citing sirtuin-3, a mitochondrial deacetylase, which regulates key processes related to aging and neurodegeneration.[60]

Our pilot study has some limitations to acknowledge. First, the sample size of the study is relatively small, and larger well-controlled studies are required to confirm these pilot findings. In addition, the use of nonglaucomatous controls, as in other studies, did not exclude cataract diagnosis from enrolment. However, cataract subjects are normally enrolled in comparative studies as among the different eye diseases, cataract subjects are expected to be overall comparable with the healthy population and it is uncommon to have age-appropriate persons without cataracts as the majority of a population may be expected to have the formation of cataracts at or above age 65 where POAG disease is common (https://www.nei.nih.gov/learn-about-eye-health/eye-health-data-and-statistics/cataract-data-and-statistics/cataract-tables).

Our study also does not analyze the phenotypical characterization of the subjects or whether the alterations reported are common to different blood cell types or of selected populations (e.g., monocytes or lymphocytes). The POAG and nonglaucomatous control subjects enrolled in this analysis, therefore, may have limited applicability to certain other glaucoma populations, including those of African descent. Our study design did not include longitudinal data, thereby limiting the commentary on the link of our results to disease progression. Significantly larger sample studies with longitudinal endpoints in diverse patient populations are needed to understand the mechanistic contributions of these findings to POAG disease and its associated vision loss over time.

Furthermore, whether the alterations here identified are specific to glaucoma or, in general, to neurodegenerative processes must be addressed by enrolling additional populations diagnosed with neurodegenerative processes of the retina and the brain other than glaucoma.

In view of this, our findings should be interpreted as "a proof-of-concept" confirming that in humans, like rodents, an eye disease, like glaucoma, may be characterized by complex and heterogeneous molecular alterations of immune cells, as robustly introduced by the pioneering results of Petriti and co-workers.[19]

Future well-controlled longitudinal studies, with a phenotypical characterization of individual cell types to run cell-specific proteomics assays and a more specific investigation of pathways uncovered as dysregulated, are required to confirm these pilot findings and further elucidate their impact on POAG disease onset and progression.

4. MATERIALS AND METHODS

4.1. Subjects' Selection and Enrollment. Peripheral blood mononuclear cells (PBCMs) were isolated in subjects diagnosed with POAG, and control patients admitted to IRCCS-Fondazione Bietti over >12 months. All procedures were approved by the local ethical committee and performed in accordance with the declaration of Helsinki and the Good Clinical and Laboratory Practices. Enrolled subjects were asked to read and sign the written informed consent prior to study participation. A detailed medical history was asked to ascertain whether subjects were eligible for enrollment in the study according to the exclusion criteria summarized below.

Inclusion criteria for POAG subjects included the following: age > 18 years and confirmed diagnosis of POAG based on European Glaucoma Society criteria. Nonglaucomatous controls were aged >18 years and diagnosis of cataract was accepted, as done in other studies.

Exclusion criteria included comorbidities and neurodegenerative disorders, including pseudoexfoliation and pigmentary glaucoma as well as angle closure and congenital glaucomas, cancer, immune system and metabolic syndromes, infectious diseases, use of glucocorticoids, or immunosuppressive drugs.

A detailed medical history was collected at the time of the patient's first visit and was reconfirmed or updated during follow-up visits. The medical history of each of the patients enrolled in this study was thoroughly reviewed to exclude systemic diseases that could potentially affect the analysis; some of the patients who underwent surgery (see Table S1) were further tested before the procedure, and no previously undiagnosed diseases were found.

Demographic, epidemiological, and clinical parameters of enrolled subjects, including the list of systemic and topical medications, are summarized in Table S1.

4.2. PBMCs Isolation Procedure. Venous blood was sampled in 10.0 mL of Becton Dickinson (BD) Vacutainer blood collection tubes containing ethylenediamintetraacetic acid (EDTA). PBMCs were freshly isolated within 1 h from sampling by density-gradient centrifugation using Ficoll–Histopaque (FH) (Merck, Darmstadt, Germany). Briefly, whole blood was first diluted 1:1 in sterile 1× phosphate-buffered saline (PBS) and then gently layered on top of FH (1:1 FH-blood ratio). Tubes were then centrifuged at 1500 rpm, 30 min, at room temperature (rt), with the brake set to

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off. After centrifugation, the PBMCs layer was gently isolated, washed three times with 1× PBS (1500 rpm, 10 min, rt), counted by Trypan blue staining using a Thoma cell counting chamber, and stored at −80 °C until use.

4.3. Shotgun Proteomics Workflow. The profiling of the PBMCs proteome was undertaken by shotgun/bottom-up proteomic workflows under LFQ. PBMCs pellets of n = 12 subjects per experimental group (Table S1 and Supporting Information 1) were lysed in prechilled urea denaturing buffer (8 M urea, 50 mM Tris-HCl, 150 mM NaCl, pH 8) supplemented with a proteases inhibitors cocktail, PMSF and phosphatases inhibitors (sodium orthovanadate and β-glycerophosphate, both 1 mM), sonicated (3 bursts, 10 s each, at 30 Hz) and cleared by centrifugation (13,000 rpm, 10 min, 4 °C).

Protein concentration was determined by BCA protein assay, and 200 μg was enrolled for trypsin digestion. First, proteins were denatured with 5 mM dithiothreitol (DTT) (45 min, room temperature [r.t.]) and alkylated with 10 mM iodoacetamide (30 min in the dark, r.t.). Then, samples were diluted to reduce urea concentration to 1 M and digested with mass-grade lysil C endopeptidase (Wako Chemicals, Japan) (1:200 enzyme:protein ratio, 2 h, rt) and then mass-grade trypsin (Fisher Scientific, Waltham, MA, USA) (1:20 overnight, r.t.). The reaction was quenched with 0.4% trifluoroacetic acid (TFA), and peptides were desalted and cleaned using C18 stage tips (Fisher Scientific, Waltham, MA, USA) by following the manufacturer's instructions. Peptides were quantified by BCA peptide assay to inject equal quantities for each sample, dried in a Speed Vacuum concentrator, and resuspended in 5% acetonitrile (ACN), and 0.1% formic acid (FA).

4.4. Mass Spectrometer Settings. For each sample, 1 μg of peptides were analyzed (two injections) by liquid chromatography (LC)/MS-MS using an Orbitrap Exploris 240 (Thermo Fisher Scientific, Waltham, MA, USA) online with a nano ultrahigh pressure LC system (Dionex, Ultimate 3000).

Data were acquired under three different modalities:

1) Data-independent acquisition (DIA);
2) Data-independent acquisition over a different m/z windowing to meet the general criteria for generation of an experiment specific DIA library by gas phase fractionation (GPF);
3) Data-dependent acquisition (DDA).

The parameters used for the analysis are listed in Table S4. A quality control sample (HeLa digest, Fisher Scientific, Waltham, USA) was injected every day of continuous analysis.

4.5. Western Blotting. Western blotting studies on a selected panel of proteins were run to confirm the proteomics data. To increase the robustness of data, in addition to the samples analyzed by mass spectrometry, an additional n = 14 subjects for each experimental group (Table S1) were included in the analysis (for a total of n = 26 subjects/group). Whole-cell lysates prepared as described above (urea buffer) were used. In all cases, samples were heat-denatured and reduced in Laemmli buffer 1× supplemented with 1 mM DTT. Thereafter, 4−20% acrylamide precast gels (Bio-Rad, Hercules, CA, USA) were used to separate proteins by SDS-PAGE. After separation, proteins were transferred to HyBond-ECL nitrocellulose filters (Bio-Rad, Hercules, CA, USA) and probed with the antibodies indicated. All antibodies used were purchased from Cell Signaling Technology (Danvers, Massachusetts, USA), except for the NADK antibody that was purchased from Proteintech (Rosemont, IL, USA) and Bcl-2 (Santa-Cruz Technology, CA, USA). Antibodies were diluted 1:4000 in 0.1% Tween-PBS 0.1% fat-free milk and, after, incubated with a horseradish peroxidase-conjugated antirabbit or antimouse IgG antibody (Bio-Rad, Hercules, CA, USA), diluted 1:10.000 in 0.1% Tween-PBS 0.1% fat-free milk. Bands were developed by ECL chemiluminescence and recorded in an iBright (Thermo Fisher Scientific). These parameters were used as a reference to address target proteins toward total protein normalization or normalization on classic internal controls (Sbardella et al., 2021). To obtain a good visualization of proteins, the micrograms loaded were found to fall off the linearity range of β-actin or GAPDH. Therefore, the fold change in individual proteins was calculated by normalizing the protein band intensity to the total proteins loaded in each lane. To this aim, filters were stained with Ponceau S and gels by Coomassie Brilliant Blue (CBB) (this last stain is not shown).

The dynamic linear range for internal controls (e.g., tubulin, actin, and GAPDH) was previously calculated in our lab and calculated here again providing the same plots.

Uncropped Western blotting filters are provided in Figure S4.

4.6. Bioinformatic Analysis. According to the different modalities of acquisition, raw proteomics files were analyzed by different strategies, specifically:

(1) DIA.raw files were analyzed by DIA-NN software under library-free search mode and using the UniProt database (UP000005640−9606, containing 82685 entries). By enabling the Match Between Run (MBR) and the library generation tool, an experiment-specific DIA spectral library was generated, and samples were reprocessed using this library. Data commented below are based on this output.

(2) In the case of GPF, the GPF-refined library was generated as described elsewhere using DIA-NN software.[33] Thereafter, raw files were analyzed using this library in place of that indicated in the previous point.

(3) DDA data were analyzed by Proteasome Discoverer (2.5) using the Sequest software and Peptide Spectrum Matches (PSMs) validated using a concatenated target-decoy strategy. A contaminant list was included in the analysis.

Regardless of the acquisition modality, the following parameters were used for all the searches: carbamidomethylation of cysteines was set as static modification and N-terminal acetylation of peptides and oxidation of methionine were set as variable modification. False discovery rate (FDR) was set in all cases to 1%.

4.7. Data Analysis and Statistical Analysis. A quantile normalization strategy was preferred over other approaches. A Mann−Whitney test with Benjamini−Hochberg correction for multiple comparisons was used ($p \leq 0.05$). In the case of Wb data, Student's t test was generally applied. Data analysis and visualization of data (Volcano plot, Venn diagram, GO enrichments, multivariate analysis) were done by R software (4.3.1) with the preprocessCore and clusterProfiler packages (Bolstad B (2024). preprocessCore: A collection of preprocessing functions. R package version 1.66.0)[61,62] and by

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STRING Network. GraphPad Prism software (8.0) was used for the analysis of Wb data. Significance in all cases was set at $p \leq 0.05$.

■ ASSOCIATED CONTENT

Data Availability Statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD059976 and 10.6019/PXD059976.[63]

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c10035.

Additional experimental details, materials, methods, and analysis (including raw Western blotting images) (PDF)

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Notes

The authors declare the following competing financial interest(s): A.H. received remuneration from AdOM, Qlaris, and Cipla for serving as a consultant, serves on the board of AdOM, Qlaris, and SlitLed, and holds an ownership interest in AdOM, Oxymap, Qlaris, and SlitLed. A.V.V. is an external collaborator of the Fondazione Bietti.

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■ ABBREVIATIONS

ACE: angiotensin converting enzyme
ACN: acetonitrile
ARB: angiotensin receptor blockers
BCVA: best corrected visual acuity
BP: biological process
CBB: Coomassie brilliant blue
DDA: data-dependent acquisition
DEP: differentially expressed proteins
DIA: data-independent acquisition
DTT: di-thiothreitol
EGS: European glaucoma society
FA: formic acid
FC: fold change
FDR: false discovery rate
GO: Gene Ontology
GPF: gas phase fractionation
IOP: intraocular pressure
LC: liquid chromatography
LFQ: label-free quantification
MBR: match between runs
MF: molecular function
PBMC: peripheral blood mononuclear cells
PBS: phosphate-buffered saline
PCA: principal component analysis
POAG: primary open-angle glaucoma
PSM: peptide spectrum matches
RGC: retinal ganglion cell
SD: standard deviation
TFA: trifluoroacetic acid
WB: Western blotting

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