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Whole blood transcriptomic analysis in ANCA-associated vasculitis

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Author:

Panagiotis Garantziotis, MD

Thesis supervisors:

Prof Dimitrios T.Boumpas

Prof Dimitrios Vasilopoulos

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Supervisor: Professor Dimitrios Boumpas, National Kapodistrian University of Athens, Medical School

Three-member committee of inquiry:

- **D. Boumpas**, MD and FACP, Professor and Chairman Department of Medicine, Medical School, National and Kapodestrian University of AthensAssociate Member, Biomedical Research Foundation Athens Academy, Athens GreeceMember, Academy of Europe (Academia Europaea)
- **D. Vasilopoulos**, Professor at the National Kapodistrian University of Athens, Medical School
- **P. Verginis**, Researcher C' (BRFAA)

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Περίληψη

Οι ANCA σχετιζόμενες αγγειίτιδες (AAV) είναι μια ομάδα σπάνιων αυτοάνοσων, δυνητικά απειλητικών για τη ζωή νόσων, οι οποίες μπορούν να επηρεάσουν διάφορα όργανα. Χαρακτηρίζονται από νεκρωτική φλεγμονή και καταστροφή κυρίως μικρών αγγείων. Οι AAV συσχετίζονται συνήθως με το ANCA που είναι ειδικό για τη μυελοϋπεροξειδάση (MPO) ή την πρωτεϊνάση 3 (PR3) και υπάρχουν φυλετικές / εθνοτικές και γεωγραφικές επιρροές στον επιπολασμό, στις συχνότητες των οροτύπων και στους κλινικοπαθολογικούς φαινοτύπους. Η παθογένεση των ασθενειών αυτών δεν είναι ακόμη πλήρως κατανοητή και η έλλειψη αποτελεσματικών μοριακών βιολογικών δεικτών συχνά εμποδίζει την ακριβή πρόγνωση και την επιτυχή διαστρωμάτωση των ασθενών.

Προκειμένου να βελτιωθούν τα κριτήρια ταξινόμησης και να παρουσιαστούν νέες ιδέες για τους υποκείμενους μοριακούς μηχανισμούς, παρουσιάσαμε το μεταγραφικό προφίλ του περιφερικού αίματος 42 ασθενών με AAV και 11 υγιών άτομων χρησιμοποιώντας εργαλεία ανάλυσης αλληλουχίας RNA και εμπλουτισμού. Δείξαμε ότι η υπογραφή γονιδιακής έκφρασης στις AAV χαρακτηρίζεται από εκτεταμένη απορρύθμιση των σηματοδότοδικών μονοπατιών της IFN και της αποκοκκοποίησης των ουδετερόφιλων. Έχουμε επιπλέον δείξει ότι η θετικότητα ANCA συνοδεύεται από μεταγραφικές παρεκκλίσεις σχετιζόμενες με τη μιτοφαγία που διαμεσολαβείται από το μονοπάτι της pink/parkin, τις οδούς χυμικής ανοσίας και τα γεγονότα σηματοδότησης Wnt εξαρτώμενα από τη b catenin. Οι οδούς σηματοδότησης της IFN τύπου 1 και αποκοκκιοποίησης των ουδετεροφίλων αντιπροσωπεύουν δύο ισχυρά σήματα που χαρακτηρίζουν την ενεργό νόσου. Η γονιδιακή υπογραφή που σχετίζεται με την ύφεση της νόσου απαρτίζεται από γονίδια που ρυθμίζουν τις αποκρίσεις της τύπου 1 IFN και της IFNγ και την σηματοδότηση μέσω Wnt που εξαρτάται από τη b catenin.. Η σύγκριση των μεταγραφικών προφίλ του ολικού αίματος των ασθενών με AAV σε κατάσταση ενεργού νόσου έναντι ύφεσης αποκάλυψε διαφοροποιήσεις στις οδούς σηματοδότησης της IL-10, αν και δεν υπήρχε στατιστική σημαντικότητα. Επιπλέον, ερευνήσαμε εάν συγκεκριμένες γονιδιακές υπογραφές θα μπορούσαν αποτελεσματικά να διακρίνουν υποτύπους AAV από τα υγιή άτομα. Μείωση της έκφρασης των γονιδίων που σχετίζονται με τη σηματοδότηση IFN και IFNγ τύπου 1, την αποκοκκίωση ουδετερόφιλων και με τη διαμεσολαβούμενη από RIK1 κυτταρικό θάνατο διαφοροποιούν μεταγραφικό προφίλ των GPA ασθενών από υγιείς μάρτυρες. Η υπογραφή γονιδιακής έκφρασης της MPA συσχετίστηκε με διαταραχές αποκοκκοποίησης ουδετερόφιλων και απορύθμιση των σημείων ελέγχου κυτταρικού κύκλου και των μηχανισμών απόκρισης σε βλάβη του DNA. Τα γονίδια που χαρακτηρίζουν το μεταγραφικό προφίλ του περιφερειακού αίματος της EGPA εμπλουτίστηκαν κυρίως σε μονοπάτια που σχετίζονται με τις αποκρίσεις IFN τύπου 1, τους μηχανισμούς σηματοδότησης μέσω NCAM και τους μηχανισμούς ρύθμισης των B κυττάρων. Τέλος, η PCA της ενεργού νεφρικής νόσου στην AAV και του ενεργού LN υπέδειξε ότι ξεχωριστοί παθοφυσιολογικοί μηχανισμοί διακρίνουν τις δύο κλινικές οντότητες.

Συλλογικά, τα δεδομένα μας εστιάζουν στις εκτεταμένες μεταγραφικές διαταραχές που χαρακτηρίζουν την AAV και δημιουργούν μία χρήσιμη δεξαμενή γονιδίων διαθέσιμα για περαιτέρω πειράματα. Τα πιο αυστηρά κριτήρια διαλογής των ασθενών και η ανάλυση του μεταγραφικου προφίλ κυττάρικών υποτύπων του περιφερικού αίματος μπορεί να ενισχύσουν την ειδικότητα των παρατηρήσεών μας. Τέλος, τα αποτελέσματά μας μπορούν

να συμβάλουν στην ανάπτυξη νέων μοριακών βιοδεικτών ή να βελτιώσουν την αποτελεσματικότητα των ήδη υπαρχόντων στο χαρακτηρισμό της διάγνωσης και στην καθοδήγηση της θεραπείας.

Abstract

AAV is a group of rare autoimmune, potentially life-threatening diseases, which can affect several organs. These are characterized by necrotizing inflammation and destruction of predominantly small vessels. AAV are commonly associated with ANCA specific for myeloperoxidase (MPO) or proteinase 3 (PR3) and there are racial/ethnic and geographic influences on the prevalence, serotype frequencies, and clinicopathologic phenotypes. The pathogenesis of these diseases is still not fully understood and a lack of efficient molecular biomarkers often prevents precise prognosis and successful patients' stratification.

To improve classification criteria and bring new insights into underlying molecular mechanisms, whole blood transcriptome profiling of 42 AAV and 11 healthy individuals was performed using mRNA sequencing. Herein, was demonstrated that AAV is characterized by extensive deregulation of IFN signaling and neutrophil degranulation pathways. It was further shown that ANCA positivity is accompanied by transcriptional aberrations related to pink/parkin mediated mitophagy, humoral immunity pathways and Beta catenin dependent Wnt-signaling events. Type 1 IFN signaling and neutrophil degranulation pathways represent two robust signals that characterize active disease status. A remission signature is linked to genes that regulate type 1 IFN and IFNγ responses and Wnt-mediated beta catenin signaling. Comparison of whole blood transcriptional profiles of AAV patients in active versus remission status revealed alterations in IL-10 signaling pathways, although statistical significance lacked. Furthermore, it was shown whether specific gene signatures could efficiently discriminate AAV subtypes from healthy individuals. Downregulation of genes related to type 1 IFN and IFNγ signaling, neutrophil degranulation and RIK1-mediated cell death differentiated GPA patients from healthy controls. MPA subtype was associated with neutrophil degranulation disturbancies and deregulation of cell cycle checkpoints and DNA damage response mechanisms. The gene set defing EGPA was mainly enriched in pathways related to type 1 IFN responses, NCAM signaling and B cell regulation mechanisms. Finally, PCA of AAV active renal disease and active LN implied that distinct pathophysiological mechanisms distinguish the two clinical entities.

Collectively, this study brings into focus the extensive transcriptional perturbations characterizing AAV and generated a useful resource available for further hypothesis generation and experimentation. More-strict patient inclusion criteria, deconvolution analysis and cell type specific transcriptional profiling might enhance the specificity of these observations. Finally, these results may contribute to novel molecular biomarkers development or improve the effectiveness of already existing ones in diagnosis characterization and therapy guidance.

Introduction

1.1. Classification of clinical phenotypes – Clinical features

Vasculitis is inflammation and necrosis of a blood vessel with subsequent impairment of blood flow. The vessel wall destruction leads to perforation and hemorrhage into adjacent tissues. The endothelial injury leads to thrombosis and subsequent deregulation of blood supply causing ischemia and infraction of dependent tissues. Long term consequences include accelerated atherosclerosis of involved vessel, which contributes to morbidity and mortality.

The antineutrophil cytoplasmic antibody (ANCA)-associated vasculitides (AAV) primarily affect small and medium arteries and include granulomatosis with polyangiitis (GPA, formerly Wegener granulomatosis), microscopic polyangiitis (MPA), eosinophilic granulomatosis with polyangiitis (EGPA, formerly Churg Strauss syndrome) and renal-limited vasculitis with pauci-immune necrotizing/crescentic glomerulonephritis (RLV) [1, 2].

AAV is a group of rare autoimmune, potentially life-threatening diseases, which can affect several organs. Its incidence in Europe is reported to be 13 to 20 cases per million [3]. AAV are slightly more common in men and the incidence increases with age [4, 5]. An overall peak in the 65-75 age group has been reported [3-6].

Classification criteria

They have been several attempts to establish classification criteria for AAV [7-10]. The International Chapel Hill Consensus Conference (CHCC) nomenclature, which has been revised in 2012, is one of the most commonly cited.

(A) ACR criteria

The American College of Rheumatology (ACR) 1990 classification criteria proposed diagnostic criteria to standardize the clinical research in GPA and distinguish these patients from those with other types of vasculitis [8].

The ACR criteria include the following features: 1) Nasal or oral inflammation, 2) Pathologic chest radiograph, including nodules, inflammatory infiltrates or cavitary lesions, 3) Abnormal urinary sediment (microscopic hematuria with or without red cell casts), 4) Granulomatous inflammatory process identified on biopsy of an artery or perivascular area.

ACR criteria fail to discriminate GPA from other forms of AAV or nonvasculitic mimics and function poorly as diagnostic criteria [11].

(B) CHCC criteria

The CHCC criteria are not a diagnostic tool that guides clinical management [10]. They provide definitions according to pathology, including distribution of vessel and organ system involvement. According to CHCC criteria, the absence of immune deposits suggests a major difference between GPA, MPA and EGPA from other forms systemic small-vessel vasculitis. GPA and EGPA are associated with granuloma formation and necrotizing vasculitis, whereas

those features do not characterize MPA. The CHCC nomenclature highlights the importance of ANCA serology, although it is not suggested as a diagnostic criterion.

1.1.1. Granulomatosis with polyangiitis

GPA is defined as a systemic vasculitis that clinically and histologically affects small and medium arteries. It is characterized by upper and lower respiratory tract involvement with granulomatous vasculitis of mainly small vessels, extravascular granulomatous inflammation and necrosis [12-14]. Clinical evidence of renal disease involves glomerulonephritis that is pauci-immune, focal, segmental, necrotizing and often crescentic. A strong association with c-ANCA and anti-proteinase 3 (PR3) antibodies has been reported [16-18].

Chronic inflammation of the mucosa of the upper respiratory tract results in chronic sinusitis that is a common presenting manifestation. Chronic purulent nasal discharges, epistaxis, mucosal ulcerations and less commonly perforation of the nasal septum and disruption of the supporting cartilage of the nose (saddle-nose deformity) may occur [12, 14, 19]. Pharyngeal mucosa can be also affected leading to obstruction of the auditory canal, which in turn results in suppurative otitis media, chronic serous otitis media and conductive hearing loss [19]. Finally, laryngeal and tracheal mucosa affection is associated with subglottic stenosis, which may present with stridor and respiratory insufficiency [20].

GPA patients typically present with pulmonary disease. Parenchymal lung nodules are a well characterized manifestation [19, 21]. The nodules have the tendency to cavitate centrally, resulting in chronic lung function deterioration [22]. Diffuse potentially life-threatening alveolar hemorrhage is a prominent manifestation of GPA affecting 5% to 45% of patients [23, 24]. Patients typically present with dyspnoea, hemoptysis, cough, anemia, hypoxemia, whereas constitutional symptoms often coexist. The concurrent present of alveolar hemorrhage and glomerulonephritis is considered as a form of pulmonary-renal syndrome [25]. Fibrosing interstitial lung disease (ILD) has been reported in GPA patients and in some cases clinical evidence of ILD precedes appearance of systemic vasculitis features [26, 27, 28]. Proximal pulmonary artery stenosis is a rare manifestation GPA [29].

Clinical evidence of renal disease occurs in approximately 18% of GPA patients on presentation [14]. Typical clinical features include elevation of serum creatinine accompanied by glomerular hematuria, hypertension, edema and usually subnephrotic proteinuria [14]. In more severe case, rapidly progressive glomerulonephritis may occur. Patients with progressive renal disease ultimately may experience chronic renal failure. The renal pathology is a focal, segmental necrotizing glomerulonephritis, frequently with crescents [30]. Immunofluorescence studies usually reveal little or no deposits of immunoglobulins, immunecomplexes or complement, which is the reason for pauci-immune designation [31]. Rare cases of interstitial nephritis or glomerulonephritis with glomerular immune complex deposition on immunofluorescence microscopy have been described [31, 32].

Involvement of peripheral and central nervous system occurs 11% to 65% of patients. Mononeuritis multiplex is the most common peripheral neuropathy, whereas symmetric polyneuropathy occurs less commonly [33]. Central nervous system syndromes involve cranial neuropathies, external ophthalmoplegia, sensorineural hearing loss, chronic

pachymeningitis seizures, pituitaty involvement [34], brain stem and spinal cord lesions, cerebrovascular events and brain hemorrhage.

About 50% of GPA patients have cutaneous manifestations. Lesions include leukocytoclastic angiitis, resulting in palpable purpura typically involving lower extremities, ulcers, subcutaneous nodules, urticaria and livedo reticularis [35]. The pathologic skin lesions are necrotizing vasculitis with or without granulomatous infiltration of the vessel wall, accompanied by extravascular granulomatous infiltration and necrosis.

Besides the upper and lower respiratory tract, the kidneys, the nervous system and the skin all organ systems can be affected by GPA. Ophthalmic and orbital involvement includes fibrotic infiltration of the retroorbital space (retroorbital pseudotumor), which causes proptosis and in the long term impairment of visual acuity and loss of conjugate gaze [36, 37]. Patients with GPA may also develop scleritis, episcleritis, uveitis, conjunctivitis, nasolacrimal duct obstruction or retinal vasculitis [36, 37]. Myalgias and arthralgias affect approximately 70% of patients, although synovitis and erosive arthritis are rarely seen. GPA is also characterized by fever, anorexia and weight loss [12, 14]. Cardiac involvement occurs in up to 5% of patients. Pericarditis, myocarditis or conduction system disorders are considered typical cardiac manifestations [14, 38]. Less frequently genitourinary tract (bladder/urethral vasculitis, orchitis, epididymitis, prostatitis), gastrointestinal tract (intestinal perforation), parotid glands thyroid gland or liver are affected [14, 38, 39]. Finally, in patients with GPA an increased incidence of venous thromboembolism has been described [40, 41].

Laboratory investigation should be performed in any patient, who presents with symptoms and signs suggestive of AAV. Patients with active, generalized GPA have a 90% likelihood of being ANCA positive, whereas only 60% of limited forms of GPA are ANCA positive [12, 42, 43]. Among ANCA positive GPA patients the vast majority are cytoplasmic (c)-ANCA positive with ANCA mainly directing against PR3 autoantigen [12, 43]. 10-20% of ANCA positive GPA patients are perinuclear (p)-ANCA positive with MPO specificity [12, 43]. Common abnormalities also include anemia of chronic disease, thrombocytosis, leukocytosis, elevated globulin levels and rise of erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) [14].

1.1.2. Microscopic polyangiitis

MPA is a primary vasculitis of small vessels that is characterized by focal segmental necrotizing glomerulonephritis and pulmonary capillaritis. Typically, vessel lesion does not involve immune deposits. Symptoms usually invade abruptly, although uncommonly insidious onset may occur.

The majority of MPA patients usually present with acute onset of rapidly progressive glomerulonephritis [19]. Clinical manifestation do not significantly differ from GPA associated renal disease, involving hematuria with dysmorphic red blood cells and cellular casts, a variable degree of proteinuria (usually of subnephrotic level) [44], serum creatinine elevation, abnormal arterial pressure and edema. The renal pathology is focal segmental necrotizing glomerulonephritis, frequently with crescents and the immunofluorescence and electron microscopy show no immune deposits (pauci-immune) [7, 31]. Pauci-immune

necrotizing glomerulonephritis typically coexists with extra-renal organ involvement. Additionally, renal-limited ANCA positive vasculitis (75% myeloperoxidase-ANCA) has been described [45-47]. Nevertheless, some patients diagnosed with renal-limited vasculitis, subsequently develop extra-renal features of MPA [19].

Diffuse alveolar hemorrhage is a potentially life-threatening manifestation of MPA, which occur in 20% to 30% as initial finding [48, 49]. ILD is also commonly associated with MPA [27, 28]. According to a MPA German cohort 12% of patients presented at diagnosis with ILD, whereas 15% developed ILD later in the course of the disease [48]. The most commonly appeared radiographic pattern resembled usual interstitial pneumonia (UIP) [48]. Finally, pleural effusions and pulmonary infiltrates can also be seen.

Non-specific constitutional symptoms, such as fever, arthralgias, myalgias are common complaints of MPA patients [7, 13]. Ear/nose/throat involvement, including nasal crusting, sinusitis, otitis media, purulent/bloody nasal discharge has been reported in approximately 30% of patients with MPA [12, 19]. 40% of MPA patients suffer from purpura, which is considered the most common MPA-associated skin lesion. Skin biopsy reveals leukocytoclastic vasculitis. Moreover, gastrointestinal tract has reported to be affected by MPA [39]. Peripheral nervous system disease affects 70% of MPA patients, whereas the presence of mononeuritis multiplex is associated with worse prognosis [33, 50, 51].

Evaluating a patient with suspected AAV requires laboratory investigation targeting ANCA detection. p-ANCA directed against myeloperoxidase (MPO) are detected in up to 60% of MPA cases [43, 52]. Notably approximately 20% of MPA patients have an alternative ANCA and in 10% ANCA are not found [43, 52, 53]. Additionally, the presence of inflammation markers such as normocytic, normochromic anemia, elevation of ESR and CRP, leukocytosis reflect the systemic inflammatory nature of MPA.

1.1.3. Eosinophilic granulomatosis with polyangiitis

EGPA is defined as a granulomatous vasculitis of mainly small- and medium-sized vessels. It is a multisystem disorder involving commonly lungs, upper respiratory tract, skin and peripheral nervous system, accompanied by prominent peripheral eosinophilia [10, 54-56]. A previous history of atopic manifestations is also common among those patients [57].

Three sequential clinical phases of EGPA have been described, although they do not always have to follow one another [58, 59]. The prodromal phase may persist for years and is characterized by predominantly allergic clinical features, such as rhinitis, polyposis and adult-onset asthma. Typically it follows the eosinophilic phase, which is associated with eosinophilic infiltration of several organs, mainly lung and gastrointestinal tract and peripheral blood eosinophilia. Finally, systemic vasculitis phase is accompanied by potentially lethal small and medium vessels vasculitis features, which often lead to extravascular granuloma formation.

Asthma is a central clinical manifestation of EGPA, appearing in more than 90% of patients [57, 60]. Asthma usually begins in adulthood and precedes the vasculitic phase by 5 to 10 years [56, 61]. The control of EGPA associated asthma, although sufficient treatment is poor and can suddenly deteriorate as the patient moves into vasculitic phase. Additional

pulmonary findings including patchy and shifting infiltrates, nodular infiltrates without cavitation, pleural effusion, alveolar hemorrhage and ILD are noted in 50 to 70% of cases [54].

Paranasal sinus involvement is a well characterized EGPA clinical feature. Allergic rhinitis, nasal polyposis, acute or chronic sinusitis and serous otitis are common in EGPA (50-70%) [62-64]. The EGPA patients commonly present mild-to-moderate mixed or sensorineural hearing loss [65]. Laryngeal and tracheal involvement with necrotizing lesions occurs less often in EGPA compared to GPA.

Skin involvement such as subcutaneous nodules, purpura or skin infarction is considered one of the most commonly seen features of the vasculitic phase [54, 66]. Extensor surfaces of the arms and legs are often affected. Skin biopsies of the lesions often show granulomas.

Cardiovascular implications of EGPA are serious and if left untreated, they can lead to death [67]. Cardiac abnormalities include myocarditis, pericarditis and valvular insufficiency, whereas heart failure was noted in 45% to 70% of patients with EGPA associated cardiac disease [57, 67-69]. The presence of cardiac features is linked with higher peripheral blood eosiniphil counts and lower likelihood of ANCA positivity [67, 70].

Mononeuritis multiplex or asymmetric sensorimotor polyneuropathy occurs commonly in vasculitic phase and approximately 75% of patients are affected [54, 56, 69, 71-73]. Central nervous system or cranial nerve involvement is only rarely seen, although central nervous system disease suggests a poor prognostic factor in EGPA.

Patients, mainly at eosinophilic phase can develop eosinophilic gastroenteritis, which commonly manifest as abdominal pain, bloody diarrhea and colitis [58]. Renal function of EGPA patients should always be evaluated. According to a large cohort study, renal involvement occurs in 22% of cases, whereas ANCA positive EGPA patients have significantly more frequent EGPA associated renal disease [57]. Myalgias and polyarthralgias affect approximately 50% of patients, although clinically overt arthritis is a relatively rare manifestation [56]. Finally, eosinophilic lymphadenopathy occurs at 30% to 40% of patients, according to studies [74].

The characteristic laboratory abnormality of EGPA is the peripheral blood eosinophilia, which may be accompanied by tissue eosinophilic infiltration [58, 75]. ANCAs are present in 30% to 60% of patients [60, 61, 76]. These are directed primarily against MPO and are associated with a p-ANCA pattern [69, 77, 78]. ANCA positive patients are more likely to develop renal disease, mononeuritis multiplex, whereas a negative ANCA was associated with cardiac involvement [57, 60, 76]. A rise of acute phase reactants is commonly observed in EGPA suggests an unspecific indicator of the underlying inflammatory process. Elevated IgE levels can be found in a large number of patients. Finally, characteristic histopathologic findings in EGPA are small necrotizing granulomas, usually at extravascular sites with a central eosinophilic core along with necrotizing vasculitis of small vessels [79].

1.2 Pathogenesis of AAV

1.2.1 Pathogenicity of ANCA

1.2.1.1 ANCA definition

ANCAs are antibodies directed against specific proteins in granules in the cytoplasm of neutrophils and lysosomal proteins of monocytes. ANCA are present in the sera of patients with several underlying diseases. When alcohol-fixed neutrophils are used as an antigen source in indirect immunofluorescence tests, three ANCA categories may be detected according to the resulting pattern. Cytoplasmic (c) ANCA is characterized by diffuse staining of neutrophil cytoplasm. The protein recognized by c-ANCA is nearly always proteinase-3 (PR3), a proteinase in azurophilic granules of neutrophils. Perinuclear (p) ANCA result in perinuclear cytoplasmic staining. The protein recognized by p-ANCA is often myeloperoxidase (MPO) and less commonly is elastase or other proteins (lactoferrin, cathepsin G) within primary azurophilic granules of neutrophils. Atypical ANCA is the term of patterns not clearly c-ANCA or p-ANCA. The protein target of atypical ANCA is usually unclear, but in many cases is common to p-ANCA.

1.2.1.2 Evidence for the pathogenicity of ANCA

Clinical and epidemiological observations highlight the crucial role of ANCAs in the pathogenesis of AAV. ANCAs are strongly associated with the several AAV clinical phenotypes and in some cases ANCA titers correlate with disease activity. Furthermore, cases of neonatal kidney disease and alveolar hemorrhage induced by transplacental transfer of ANCA have been reported [91]. The clinical response of AAV to rituximab treatment (B cells targeted) and the beneficial effect of plasma-exchange therapy in severe forms of AAV further support the key role of ANCA in disease's mechanisms [92, 93].

Along the same lines, in vitro experiments also provide evidence of the pathogenetic contribution of ANCA. Firstly, ANCA activate neutrophils, which are primed by proinflammatory cytokines. Those ANCA-activated neutrophils are capable of causing endothelial injury and stimulating alternative complement pathway cascade, which partially facilitate the perpetuation of the inflammatory process.

Several previous in vivo studies have demonstrated the pathogenetic importance of ANCA. Xiao H et al. reported that passive transfer of mouse anti-MPO antibodies into wild-type mice or mice deficient in recombination activation gene 2 (Rag2) gave rise to severe glomerulonephritis and pulmonary hemorrhage [94]. These results suggest that anti-MPO antibodies alone can induce pauci-immune vasculitis. Taking a step forward, depletion of neutrophils prior infusion of mouse anti-MPO antibodies prevents development of anti-MPO IgG mediated vasculitis phenotype in those mice [95]. In vivo studies in rat model of ANCA-associated experimental autoimmune vasculitis (EAV) also highlighted the influence of ANCA on the pathologic leukocyte-endothelial interactions, which characterize microvascular inflammation [96]. Briefly, these rats, after immunization with human MPO generated antibodies against rat MPO, developed small vessel vasculitis and showed enhanced adhesion and migration of leukocytes on endothelial cells in response to CXC ligand 1 (CXCL1) [96]. Additionally, increased adhesion and transmigration at the endothelium upon

CXCL1 administration and similar microvascular manifestations were observed in wild type rats after passive transfer of anti-MPO from rats with EAV [96].

Taken together, clinical and experimental data suggest that ANCA are largely instrumental in initiation and development of AAV. ANCA facilitate activation of primed neutrophils and induction of endothelial transmigration of leukocytes, although the exact underlying mechanisms remain unclear.

1.2.1.3 Genesis of ANCA autoimmunity

It is not fully understood how ANCA contributes to the development of AAV. Several genetic and environmental factors have been proposed to play a causative role in the onset induction and progression of AAV. Those factors influencing AAV pathogenesis, including infection, medication, dysregulated immune responses or abnormal expression of autoantigens are quite heterogenous and differ among patients.

Circulating ANCA, often called 'natural' autoantibodies have been also detected in apparently healthy individuals [80-84]. ANCA titer difference between patients and healthy, along with qualitative alterations, such as lower avidity, less subclass diversity or reduced neutrophil-activating potential of natural ANCA may partially explain the difference in disease status [82]. Additionally, epitope spreading may favor pathogenetic ANCA formation. The capability of pathologic ANCA associated with active disease to recognize a larger number of epitopes, distinguishes them from natural ANCA and ANCA found in remission, which recognize only few epitopes [83].

Exposure to exogenous antigens, such as drugs or microbial particles is considered a contributory mechanism for pathogenetic ANCA genesis. Clinical observations have highlighted the presumable contribution of infectious episodes in the disease initiation and the flares of AAV [86, 87]. Early studies have demonstrated that chronic nasal carriage of Staphylococcus aureus is a major risk factor for relapse in GPA in conjunction with persistence of ANCA. Immune responses against infectious pathogens, such as Staphylococcus aureus may induce a modulation of epitope specificity leading to pathogenetic transformation of natural ANCA [83, 85, 86]. Antibodies against the protein translated from antisense strand of PR3 gene, known as complementary PR3 have been isolated from serum of PR3 positive patients. Taking a step forward, this complementary protein show a homology with many proteins derived from microbes, including Staphylococcus aureus peptides [84-86]. It is hypothesized, that a staphylococcal infection may lead to production of antibodies cross-reacting with complementary PR3, which finally stimulates the generation of PR3 antibodies based on idiotypic-anti-idiotypic interaction [88].

Microbial molecular mimicry seems also to provoke the production of ANCAs with specificity for LAMP-2, which in turn could play a role in disease induction [89]. Rats immunized with bacterial adhesin FimH create antibodies against rat and human LAMP-2, which are associated with glomerulonephritis similar to human pauci-immune ANCA-related glomerulonephritis [89]. Additionally, human LAMP-2 epitope shows a high homology with FimH [89]. Both observations imply that immune responses against FimH are stimulus of antibody production against human LAMP-2 and trigger AAV clinical manifestations.

Drugs, including hydralazine, minocycline, propylthiouracil and levamisole-adulterated cocaine are also hypothesized to play a role in the generation of pathogenetic ANCA [90]. Hydralazine through inhibition of DNA methylation is believed to remove the epigenetic silencing of MPO and PR3, leading to up-regulation of their expression in neutrophils [90]. This abnormal overexpression of MPO and PR3 could either promote the disruption of immune tolerance against these autoantigens or aid ANCA-mediated activation of neutrophils. Taken together, microbial factors and drugs may be involved in AAV though induction of ANCA or antibodies related to ANCA, but further investigation is necessary.

1.2.2. Pathogenetic mechanisms of vascular inflammation

1.2.2.1 ANCA-mediated activation of neutrophils and monocytes

Experimental data support the notion that ANCA-induced activation of both neutrophils and monocytes is one of the main pathogenic mechanisms involved in disease induction. According to in vitro studies, MPO and PR3-ANCA are capable of activating primed neutrophils that have been primed with tumor necrosis factor (TNF), bacterial lipopolysaccharide (LPS), or complement (C5a) [97-101]. Priming of neutrophils by proinflammatory cytokines, often released as a result of local infection (chronic nasal carriage of Staphylococcus aureus) induces an increased expression of neutrophil adhesion molecules, along with concurrent translocation of intracellular PR3/MPO at neutrophils' surface [97-101]. Those ANCA antigens interact then with circulating ANCA autoantibodies.

Taking a step forward, engagement of Fab portion of ANCA to ANCA antigens expressed on neutreuphils' surface and interaction of neutrophils' Fcγ receptors (FcγRs) with immunecomplexes formed by ANCA and antigen lead to full neutrophil activation [101-105]. ANCA binding to neutrophils stimulates intracellular pathways, which facilitate enhanced neutrophil-vessel wall adherence and transmigration [106]. Activated neutrophils undergo respiratory burst with generation of reactive oxygen species (ROS), degranulate and extrude neutrophil extracellular traps (NETs) [99-103]. These mechanisms contribute to endothelial injury and death that characterize AAV [107-109].

NETosis is a neutrophil-related cell death characterized by extracellular, web-like structures composed by cytosolic and neutrophil granular proteins captured on backbone of chromatin. ANCA are a well defined trigger of NETosis and high levels of NETs are commonly found at the sites of inflammation and in circulation in AAV [110, 111]. According to studies, NETs are involved in the pathogenesis of AAV through promoting activation of autoreactive B-cells and dendritic cells (DCs), causing endothelial damage and initiating complement cascade [112, 113]. It is also hypothesized that NETs, through transfer of neutrophilic antigens to DCs might facilitate loss of tolerance towards ANCA antigens and trigger pathogenetic ANCA generation [114].

ANCA antigens are also expressed by monocytes, suggesting that these cells may be implicated in disease pathogenesis. Similar to neutrophils, engagement of monocytes' FcγRs by ANCA serves as a stimulus, which causes monocyte activation [115, 116]. In vitro ANCA-activated monocytes secrete high amounts of proinflammatory cytokines like monocyte chemoattractant protein-1 (MCP-1) or interleukin-8 (IL-8) [115, 116]. Cytokine release in turn promotes further recruitment and activation of neutrophils at the area of inflammation

and might favor granuloma formation through monocytes chemotaxis. Additionally, expression of adhesion molecules is increased on circulating monocytes from patients with active GPA compared to healthy individuals [117]. Histologic analysis of renal lesions in AAV has revealed that monocyte is the predominant cell type in the glomeruli, suggesting that monocytes might play major in tissue damage [118].

In summary, circulating neutrophils and monocytes primed by inflammatory cytokines and C5a can be activated by ANCA. Activated leukocytes penetrate vessel wall and through degranulation, NETosis, apoptosis, necrosis and complement activation initiate and amplify extravascular inflammatory processes in AAV.

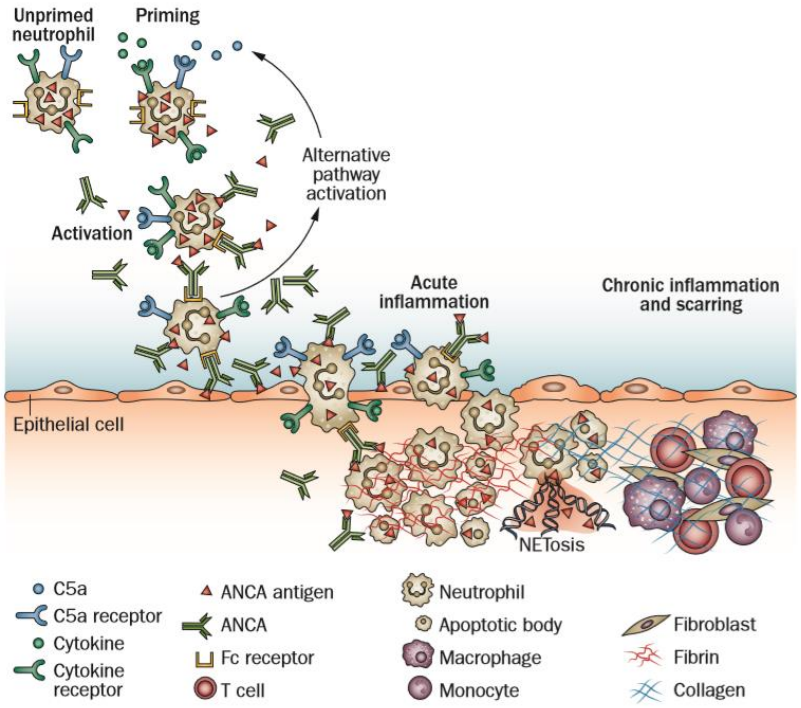


Figure 1. Pathogenetic events in AAV (Jennette JC, et al. Nat Rev Rheumatol. 2014)

1.2.2.2 The role of complement in AAV

The role of specific complement pathways in AAV has been extensively investigated in vivo. Specifically, infusion of mouse anti-MPO IgG in mice with knockout of the C4 complement component (lectin pathway component) did not prevent development of pauci-immune necrotizing crescentic gleomerulonephritis [119]. On the other hand, $C5^{-/-}$ (classic pathway) and factor $B^{-/-}$ (alternative pathway) mice did not produce vasculitic phenotype after mouse anti-MPO injection [119]. These results support the concept that alternative rather classic complement pathway is implicated in disease.

Silencing of C5aR in mice had a protective effect over disease development, whereas administration of avacopan (antagonist of human C5aR/CD88) in knock-in mice of the human C5aR/CD88 improved anti-MPO induced disease [120, 121]. Notably, mice deficient for C5a-likereceptor (C5L2) developed more severe disease after anti-MPO injection compared to wild-type mice that received mouse anti-MPO [121]. To sum up, results indicate that C5a-C5aR/CD88 interaction increases inflammation, whereas C5L2 engagement

ameliorates disease phenotype in anti-MPO induced crescentic glomerulonephritis mice model.

Complement component C5a is capable of attracting and priming circulating neutrophils, which in turn can be activated by ANCA [97-101]. However, NETs or cytokines released by ANCA-activated neutrophils can initiate alternative complement pathway cascade producing C5a [113, 119]. The sequence of events described previously can be represented as positive feedback loop, which amplifies inflammatory processes in AAV.

Studies on human renal biopsies have highlighted a relationship between increased glomerular complement deposition and pathological severity of the disease [122]. Being more specific, in glomeruli of AAV patients with active pauci-immune glomerulonephritis increased deposition of Bb was detected and associated with pathological progression of renal damage [122]. This finding proposed participation of alternative pathway activation in renal disease of human AAV.

1.2.2.3 The role of B cell in AAV

Dysregulation of B cell function has a critical role in initiation and progression of AAV. B cells produce pathogenetic ANCA, whereas B cells activation is associated with active disease status [123]. Furthermore, B cell targeted therapy (rituximab) can induce and maintain remission in many cases of AAV and appears to be effective in relapsing disease [124]. Patients in remission experiencing B cell return within 12 months after withdrawal of rituximab maintenance treatment seems to relapse earlier than those whose B cell repopulation occurred later [125]. Specifically, repopulation with low percentage of CD5+ B cells, after B cell depletion in combination with low maintenance immunosuppressive treatment has been proposed as a risk factor for earlier relapse [126]. Several studies of peripheral blood B cells in AAV have revealed differences from normal B cells. Alteration of CD19 expression seems to be a potential mechanism for causing a dysregulation of BCR signaling that may contribute to autoimmunity [127]. Although naïve B cells in AAV express significantly less CD19 compared to healthy B cells, a subset of memory B cells in AAV over-expresses CD19. This subpopulation of memory B cells is autoreactive and it likely plays a role in the course of the disease [127].

Regulatory B cells (Breg) have immunosuppressive properties, are characterized by IL-10 production and influence regulation of T cell mediated immunity [128]. Breg cells are decreased in patients with active AAV [128]. Additionally, Breg mediated suppression of Th1 responses might be inadequate in AAV [128].

BAFF (B-cell activating factor of the TNF family) is a soluble factor, which promotes B cell survival. According to in vitro experiments, ANCA-activated neutrophils secrete BAFF [131]. Increased BAFF levels have been found in serum of patients with AAV [129]. Elevated BAFF levels also have been linked with active disease and ANCA titers, although a correlation with ANCA positivity has not been reported [129, 130]. An increase in serum BAFF levels have been observed after treatment with rituximab in AAV patients [131]. Moreover, a BAFF regulatory region single nucleotide polymorphism has been proposed as a risk factor for relapse and earlier B cell repopulation after rituximab therapy, suggesting BAFF inhibition as a potential therapeutic target in selected cases after rituximab induction treatment [132].

1.2.2.4 The role of T cell in AAV

Regulatory T cells (Treg) are a subset of T cells that suppresses immune responses and contributes to maintenance of homeostasis and self-tolerance. AAV is characterized by impairment of Treg suppressive function, which is more pronounced in those with most active disease [133, 134]. However, the frequency of Treg from periphery of active AAV patients was elevated [134]. Furthermore, a subpopulation of CD4+ T cells resistant to Treg-mediated suppression was identified in a group of patients with active AAV [134]. On the other hand, increased expression of the negative co-stimulator programmed death receptor-1 (PD-1) on peripheral blood Th cells from GPA compared to health controls, might try to counterbalance auto-reactive T cells [135]. Interestingly, kidney-infiltrating T cells from the same patients mostly lacked PD-1, suggesting that PD-1 pathway might fail to regulate renal inflammation sufficiently [135].

The role of Th_{17} axis in the pathogenesis of AAV has also emerged. Serum levels of IL-17A and IL-23 were increased in patients with active AVV compared to healthy individuals [136]. Moreover, higher levels of IL-23 were linked with more active disease and higher ANCA titers [136]. One study has also shown that activated peritoneal neutrophils from ANCA-induced systemic vasculitis model are capable of producing IL-17A in response to MPO-ANCA [137, 138]. In vitro cultured peritoneal neutrophils had also the ability to secrete IL-6 and IL-27, which further facilitate amplification of Th_{17} responses [137]. Finally, deficiency of IL-17A prevented development of glomerulonephritis in murine anti-MPO–induced glomerulonephritis model [138].

1.2.3. Pathogenetic mechanisms of granuloma formation

GPA, in comparison to MPA is characterized by extravascular granulomatosis, although the pathogenetic mechanisms underlying granuloma formation are not fully understood. It is hypothesized that type-IV adaptive immune responses are involved, even though innate immunity dependent responses of macrophages might also play a role [140]. Briefly, initially granulomas consist mainly of neutrophilic micro-abscesses, whereas later granulomas are formed mostly by multinucleated giant cells, DCs, lymphocytes and plasma cells [140]. Translational studies provide weak indication that Th_1 immune responses orchestrate granulomatous inflammation [141]. Finally, study of mucosal microenvironment of GPA patients revealed the presence of chronically activated B cell, cells expressing PR3 autoantigen and increased levels of BAFF. This observation suggests that locally PR3 abundance might serve as an immunological stimulus to initiate or perpetuate chronic anti-PR3 responses [142].

1.3 Gene expression studies in AAV

Genomic approaches provide insights into molecular mechanisms underlying complex diseases. Regarding AVV, Grayson et al. performed whole-blood and peripheral blood mononuclear cell (PBMC) transcriptomic analysis to study gene expression profile of AAV patients participating in RAVE trial [143]. Specifically, patients who did not meet the primary outcome of disease remission off glucocorticoids 6 month after enrollment (non-responders) were characterized by a granulocytic whole blood transcriptional gene signature at baseline visit compared to responders. Additionally, this gene expression signature at baseline, in contrast to clinical manifestation and ANCA titers could effectively predict response to

treatment. Enrichment analysis of these differentially expressed genes (DEGs) highlighted an up-regulation of pathways involved in bacterial defense, neutrophil activation and myeloid differentiation in non-responders. Furthermore, hierarchical clustering of DEGs revealed a cluster of 179 genes, which mainly related to granulocytic properties. Interestingly, MPO and PR3, the most important auto-antigens implicated in AAV were included in this cluster. Finally, authors tried to determine whether this gene signature derives from low-density granulocytes. Among granulocyte gene signature, PR3 expression was increased in PBMCs of non-responders compared to responders, whereas transcriptional differences of MPO and CAMP were not detected.

Cheadle et al. performed a gene expression microarrays experiment using total RNA from PBMCs and neutrophil fractions to detect gene expression profiles that differentiate GPA patients from healthy individuals [144]. The authors proposed a GPA-specific signature associated with myeloid differentiation, including genes encoding ANCA auto-antigens. Noteworthy, this signature did not characterize granulocytic fraction of GPA patients. Moreover, gene expression in PBMCs of interferon-induced genes did not differ between GPA patients and healthy controls. This study has also shown that the proposed GPA signature was significantly up-regulated in patients with active disease compared to patients with inactive disease. Increased PR3 expression was detected in PBMCs from GPA patients and was associated with GPA signature.

Lyons et al. has studied transcriptional differences between systemic lupus erythematosus (SLE) and AAV [145]. PBMCs arrays data revealed type 1 interferon and plasmablast SLE-specific signatures, which could discriminate SLE and AVV. On the other hand, granulopoiesis typically characterized the transcriptional profile of both SLE and AAV. The authors also investigated the gene expression profile of purified cell subtypes from periphery of the same patients. Briefly, a T-cell activation signature, including predominantly interferon-inducible transcripts was found in CD4+ cells from SLE patients, but not in AAV patients. Furthermore, up-regulation of type 1 interferon inducible genes in SLE monocytes clearly differentiates them from AAV monocytes.

1.4 Aim of the study

Our hypothesis is that patients with AAV can be discriminated at transcriptional level when compared to healthy controls using RNA sequencing analysis. Our study aims to investigate whether subtypes of AAV are accompanied by specifc gene expression signatures. We will also attempt to investigate the possible presence of molecular disease signatures and perform inter-disease analysis. Our transcriptomic analysis will provide insights into the molecular mechanisms underlying disease development and will examine the pathogenetic role of ANCA. Along the same lines, our results may contribute to the development of potential biomarkers and be instrumental in achieving a more thorough AAV patients' stratification.

Materials and Methods

2.1 Patients and controls

We performed a case-control study. From a cohort of vascultis patients, at the Departments of Rheumatology and Clinical Immunology at Hippokration and Attikon University Hospitals, we selected three groups of vasculitis patients: 1) Granulomatosis with Polyangiitis patients (N=22), 2) Eosinophilic granulomatosis with polyangiitis patients (N=12), 3) Microscopic polyangiitis patients (N=8) (Table 1.). Eleven age and sex matched apparently healthy adults were used as control. All subjects enrolled in this study provided informed consent. The study was approved by the Ethics Committee of Attikon and Hippokration General Hospitals of National and Kapodistrian University of Athens.

2.2 Clinical definitions

AAV patients were classified according to CHCC criteria [10]. Disease activity was assessed according to BVAS/WG and the physician's global assessment [146]. Remission was defined as BVAS/WG of 0 and successful completion of the prednisone taper at 6 months after beginning of induction treatment. A disease flare was defined as an increase in the BVAS/WG of 1 point or more. Severe disease denoted a condition that threatened the patient's life or the function of a vital organ (eg, rapidly progressive glomerulonephritis, alveolar hemorrhage and mononeuritis multiplex), whereas limited disease did not pose such threats (eg, sinusitis, lung nodules, or joints involvement).

2.3 RNA sequencing experiment

The pipeline of the RNA sequencing experiment is shown in Figure 2.

2.3.1 Isolation of total RNA-Library preparation-Sequencing

Whole blood samples were collected in PaxGene and Tempus RNA tubes. Total RNA was extracted using the Qiagen RNeasy kit and quantification was assessed using a NanoDrop spectometer. Quality control of RNA was assesed using the Agilent Bio Analyser. mRNA libraries were prepared using the Illumina TruSeq kit. 1x75bp single-end mRNA sequencing was performed on the Illumina instrument in the BRFAA Greek Genome Centre.

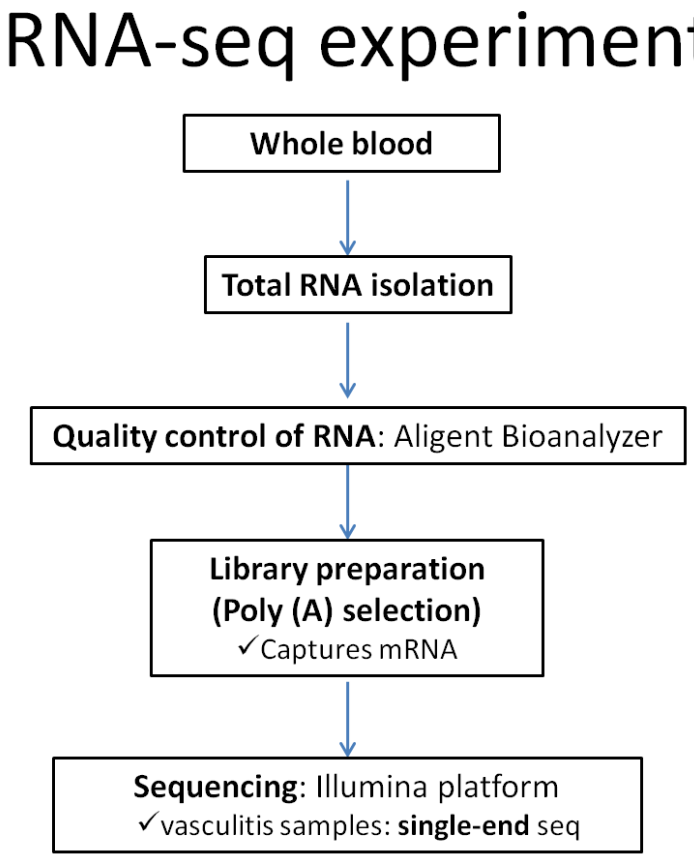


Figure 2. RNA sequencing experiment.

2.4 Bioinformatice analysis

The pipeline of the bioinformatic analysis is shown in Figure 3.

2.4.1 Quality control of raw data

Quality problems typically originate either in the sequencing itself or in the preceding library preparation. They include low-confidence bases, sequence-specific bias, 3'/5' positional bias, polymerase chain reaction (PCR) artifacts, untrimmed adapters and sequence contamination. These problems can affect mapping efficiency, assembly and expression estimates. Raw data were collected from sequencer in fastq format and the FastQC tool was used to assess the different quality aspects of the raw sequencing reads [173].

2.4.2 Data trimming

If low-quality bases (Q<30) are detected in the ends of the reads, we removed them by trimming a given number of bases from 3' end. We also removed adapter sequences from the end of the reads. Cutadapt was used to perform trimming [147].

2.4.3 Aligning Reads to Reference Genome

Reads were mapped to the reference human genome (GRCh38.p12) by STAR (Spliced Transcripts Alignment to a Reference) mapper [148]. STAR performs unbiased search for spliced junctions and can cope with long reads. The percentage of mapped reads is an important mapping quality indicator. Samples with a percentage of uniquely mapped reads of less than 50% were excluded.

2.4.4 Quantification of gene expression

In this step, a single read is associated with a gene based o the mapping location. High-throughput sequencing (HTSeq) tool was used for gene quantification [149] using Gencode v29 annotation. The output is a table of reads for each gene.

2.4.5 Differential expression analysis

We used the edgeR Bioconductor package to determine differential gene expression[150]. edgeR is based on the concept of generalized linear models. This package receives as input raw read counts and introduces potential bias sources into the statistical model in order to achieve an integrated normalization. .Statistically significant differentially expressed genes were considered those with $p<0.05$ and absolute fold change > 1.5.

2.4.6 Visualization, enrichment analysis

We used heatmap3 package in R in order to create heatmaps and perform unsupervised hierarchical clustering [151]. Enrichment analysis of DEGs was carried out to find out over- or underrepresented processes and pathways using ToppGene Suite [152]. Regulatory Network Enrichment Analysis (RNEA) was used to detect regulatory interactions from expression profiles [153]. Cytoscape software was used to visualize regulatory network constructed by RNEA and ClueGo allowed us to visualize the non-reduntant biological terms and pathways for identified DEGs in a functionally grouped network [174].

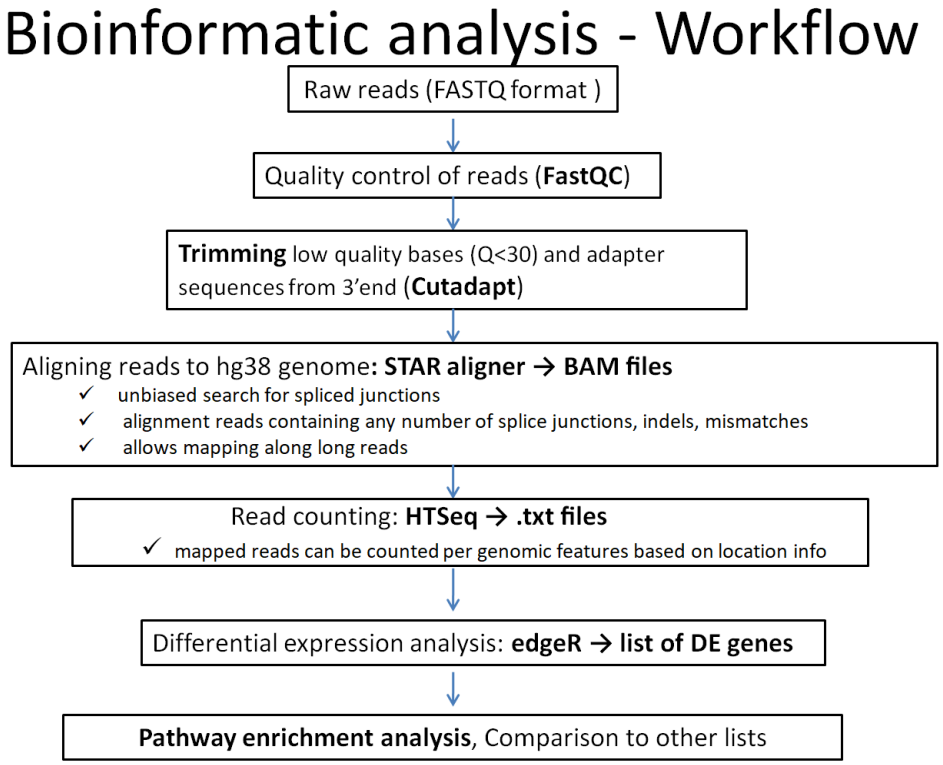


Figure 3. Pipeline of bioinformatic analysis.

Results

3.1 Sequencing characteristics

The sequencing data is characterized by a mean value of percentage of uniquely mapped reads of 70.54% and the average of the RIN of the samples is estimated to be 5.91. The mean value of the library concentration was calculated to be 2.43ng/ul and the average RNA concentration was estimated to be 99.02ng/ul. Statistically significant differences of library and RNA concentration were identified between the samples derived from Hippokration and Attikon (Supplementary 1).

3.2 Subjects characteristics

The clinical characteristics of the subject enrolled in this stude are described in Table 1. 42 AAV patients were included in the study. 52% of patients had diagnoses of GPA, 19% of MPA and 26% of EGPA. The majority of patients (75%) were ANCA positive. At the time of sampling, 19% of AAV patients were newly diagnosed, 40% were in remission, 23% had relapsing disease and 14% had persistently active disease. Renal involvement was detected in 50% of patients, although renal biopsy was required in 6 patients. Evidence of pulmonary disease was present in 88% of AAV patients, whereas upper respiratory tract involvement accounted for 51% of AAV subjects. 25% of patients had current or a history of relapsing disease. 13 patients were receiving cyclophosphamide as induction therapy at sampling, rituximab was the induction treatment in 9 cases, whereas 2 patients were treated with a combination of both. Azathioprine was chosen as a maintenance treatment in 15 patients, 5 were treated with methotrexate and 1 with rituximab after induction regime.

We also performed a longitudinal study. Clinical and transcriptomic profile of 8 patients were assessed during active disease and after remission induction. GPA was diagnosed in 5 patients, MPA in 2 patients and EGPA in1 patient. Cyclphosphamide was used to induce remission in all cases, whereas a combination of cyclophosphamide and rituximab was used as induction therapy in 2 subjects.

Table1. Prevalence of clinical features, disease activity and serological characteristics at the time of AVV diagnosis and at sampling.

Variables	All patients (N= 42)	Healthy (N= 11)
Age (years), mean (SD)		
at diagnosis	56.64(15.84)	
at sampling	60.35(15.04)	66,81(11.73)
Female Gender (n,%)	23(54.76)	4(36.36)
ANCA-associated vasculitis type (n, %)		
GPA	22(52.38)	
MPA	8(19.04)	
EGPA	12(28.57)	
Disease Status at sampling (n, %)		
Active disease, on treatment	23 (54.76)	
Remission	19 (45.23)	
Number of relapses, median* (IQR)		
Any severity	1(0-1)	
Major	0(0-1)	
Minor	0(0-1)	

BVAS/WG, median (IQR)		
at diagnosis	5(4-8)	
at sampling	1(0-3)	
Organ involvement[#] (n, %)		
Constitutional	19(54.28)	
Ear, nose and throat	18(51.42)	
Mucous membranes and eyes	4(11.42)	
Cutaneous	4(11.42)	
Pulmonary	31(88.57)	
Neurologic	8(22.85)	
Renal	20(57.14)	
Gastrointestinal	1(2.85)	
Cardiovascular	2(5.71)	
ANCA positivity at diagnosis (n, %)		
c-ANCA and/or anti-PR3	11(27.50)	
p-ANCA and/or anti-MPO	19(47.50)	
Treatment at time of sampling (n, %)		
Cyclophosphamide[†]	12(29.26)	
Rituximab[‡]	10(23.80)	
Mycophenolate mofetil	3(7.14)	
Methotrexate	5(11.90)	
Azathioprine	14(33.33)	
Prednisone	21(50)	
Prednisone dose (mg), median (IQR)	7.5(5-40)	

*from patients with history of relapsing disease

[#]during the total disease duration (from diagnosis until sampling)

[†]during the last 3 months

[‡]during the last 6 months

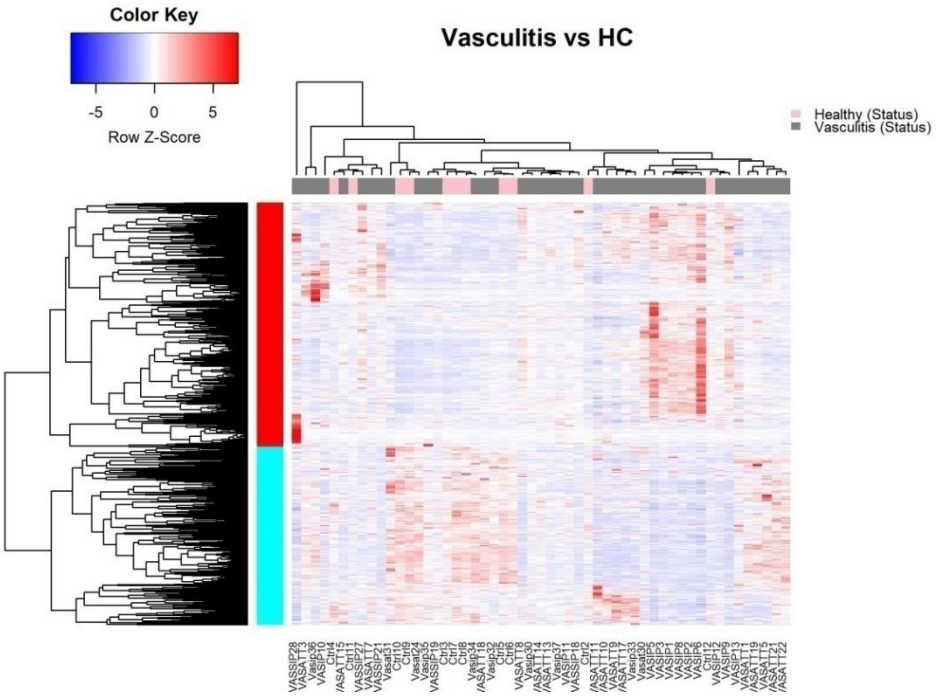
3.3 AAV whole blood transcriptome is characterized by extensive aberrancies

To acquire a deeper understanding of the biological mechanisms underlying AAV, we perform a multivariable design analysis to find out genes differentially expressed between AAV patients and healthy subjects. AAV patient pool included GPA, MPA and EGPA patients. Disease activity was not taken into account for this comparison. Principal Component Analysis (PCA) did not discriminate clearly AAV from healthy control, however healthy controls formed a distinct group on the upper right corner of the plot even if there was overlap with some patients (figure 4B-C). Differential expression analysis revealed 1,405 DEGs in AAV patients versus healthy individuals (pvalue<0.05 and absolute fold change > 1.5) (figure 4A). 803 genes were downregulated and 602 were upregulated. PPI networks corresponding with DEGs were created using RNEA and visualized using Cytoscape. Based on the degree of connectivity *LEF1, HIF1A* and *CTNNB1* were considered hub genes (figure 4C). Downregulation of interferon-related genes, such as *STAT1, IRF6, IRF7, IRF9, STAT2, USP18, IFIT1, FCGR2B* and innate immune response or inflammasome related genes, such as *HIST1H4H, ADAM8, NOD1, TRIM22, FCGR1B, FADD, NLRC5, CCL2, TRAFD1, CCL8* was observed in AAV. Pathways associated with IFNγ, type 1 IFN signaling or leukocyte differentiation were enriched (figure 4E-F).

Unsupervised hierarchical clustering analysis of DEGs identified two distinct gene clusters that efficiently differentiated AAV from healthy controls. The first cluster consists of 812

genes and the second of 563 genes. Enrichment analysis of the gene clusters confirmed previously reported perturbation in AVV transcriptome and identified novel differences in AAV compared to healthy individuals [143, 145]. Enrichment of the first gene set signature (812 genes) revealed pathways related to type 1 IFN, IFNγ and NOD-like receptor signal transduction (Table 2). Analysis also demonstrated a statistically significant enrichment of a neutrophil degranulation pathway, as in previous studies [143, 145]. On the other hand, enrichement analysis using the genes of the second cluster identified pathways associated mainly with translational processes and influenza life cycle (563 genes) (table 3). The immune-related gene signatures identified in this study suggest that type 1 interferon, IFN-γ and innate immunity deregulations are present in AAV and may contribute to disease initiation and perpetuation.

A



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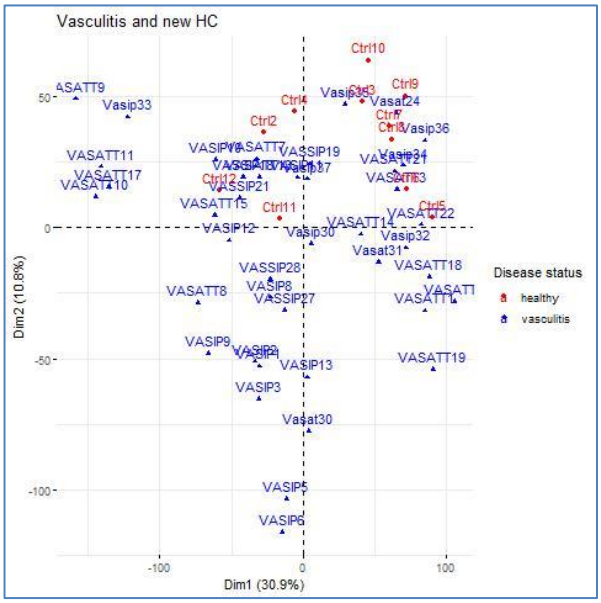

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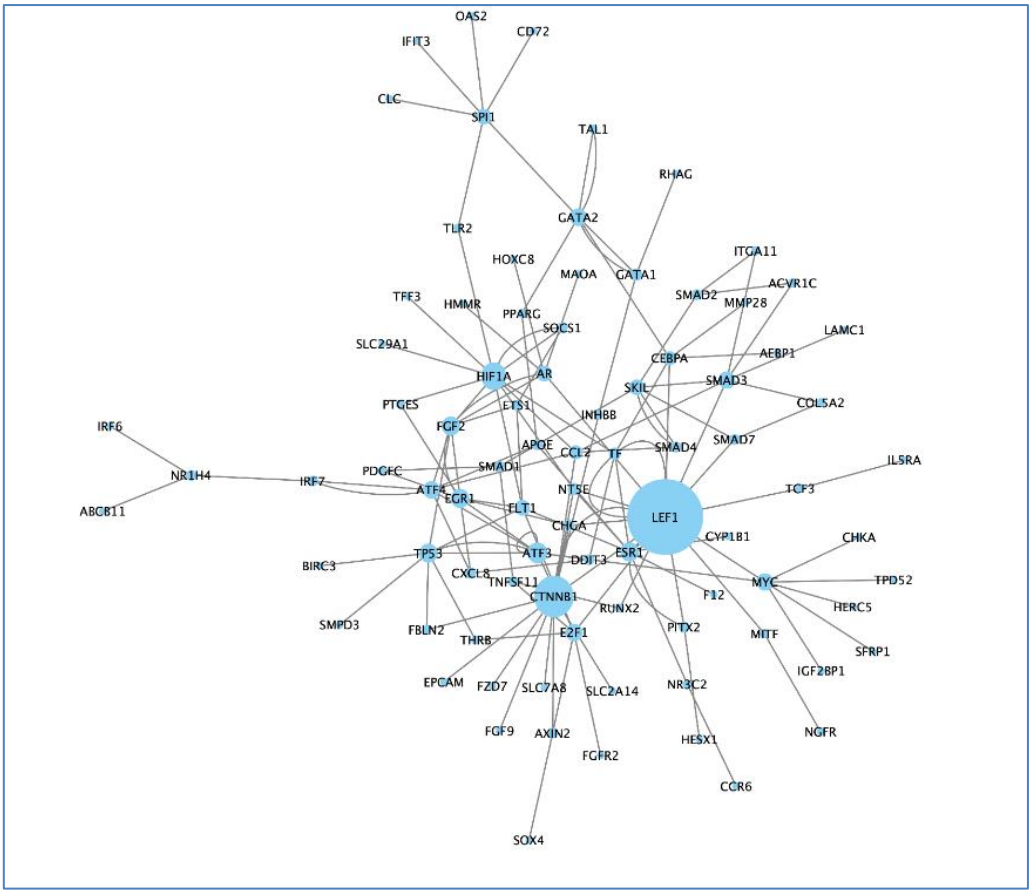
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SMAD2
MMP28
TFF3
HMMR
PPARG
SOCS1
LAMC1
SLC29A1
CEBPA
AEBP1
HIF1A
AR
SKIL
SMAD3
PTGES
ETS1
INHBB
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FGF2
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CXCL8
TNFSF11
MYC
TPD52
CTNNB1
F12
HERC5
RUNX2
SMPD3
FBLN2
THRB
E2F1
PITX2
MITF
SFRP1
IGF2BP1
EPCAM
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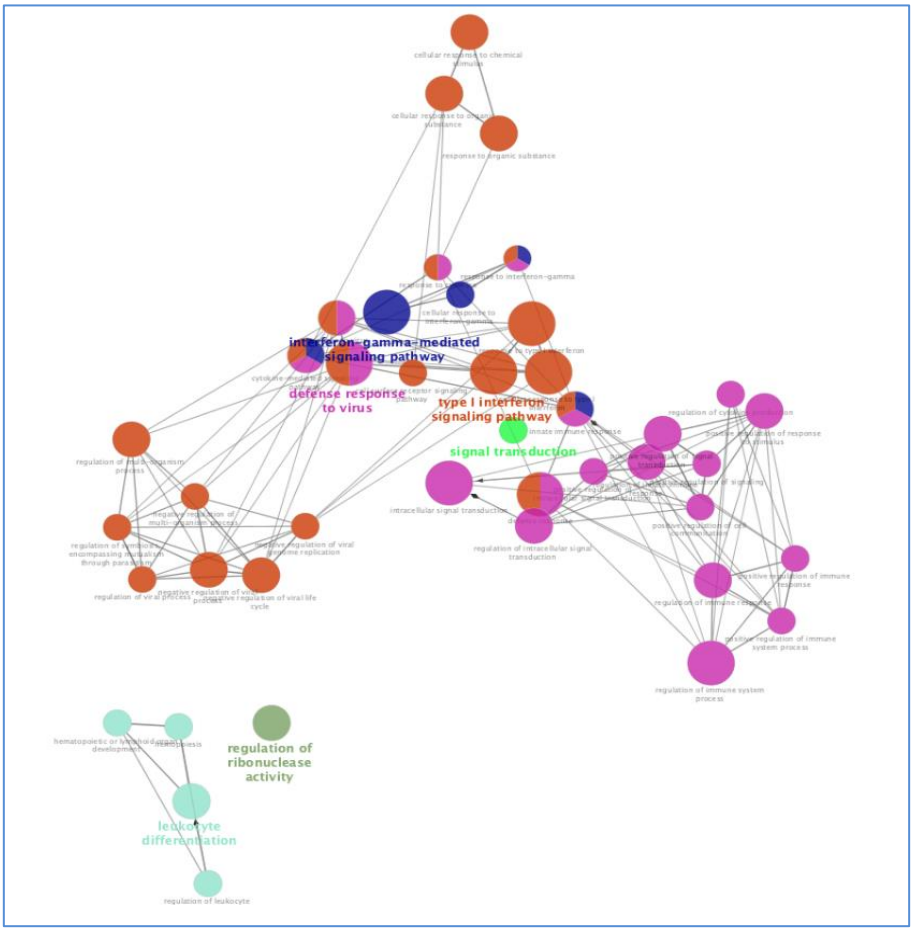

interferon-gamma-mediated signaling pathway
defense response to virus
type I interferon signaling pathway
signal transduction
regulation of ribonuclease activity
leukocyte differentiation

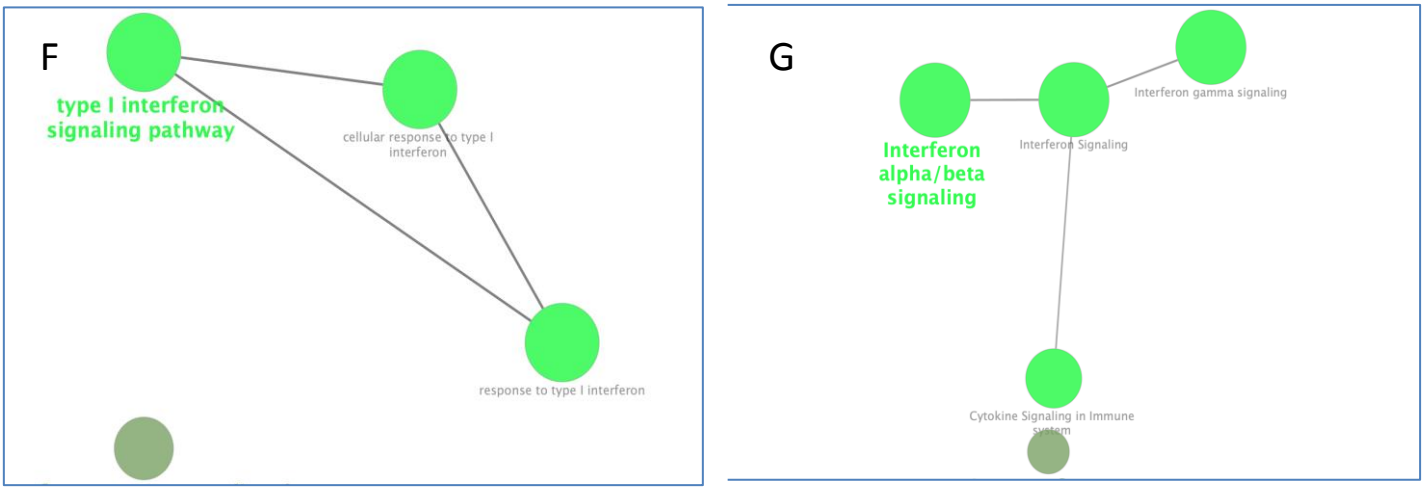


Figure 4. (A) Heatmap of DEGs in whole blood of AAV patients versus healthy individuals. (B) PCA of whole trascriptome between AAV(disease status) and healthy individuals. (C) PCA of whole trascriptome between AAV subtypes and healthy individuals. (D) PPI Network of identified DEGs in AAV versus healthy control comparison. (E) Mechanistic map of biological processes with all DEGs (AAV versus healthy control). (F)Mechanistic map of immune regulation with all DEGs (AAV versus healthy control). (G) Pathway enrichment analysis with all DEGs (AAV versus healthy control).

Table 2. Pathway enrichment analysis of a cluster of 812 DEGs in AAV versus healthy individuals (red cluster on heatmap shown in Figure 4A.).

Category	Name	Source	p-value	Hit query list	Hit Genome
Pathway	Interferon Signaling	BioSystems: REACTOME	1,45E-10	32	202
Pathway	Interferon alpha/beta signaling	BioSystems: REACTOME	6,21E-09	18	69
Pathway	Interferon gamma signaling	BioSystems: REACTOME	1,08E-05	17	94
Pathway	Innate Immune System	BioSystems: REACTOME	4,48E-05	80	1312
Pathway	Neutrophil degranulation	BioSystems: REACTOME	1,13E-03	38	492
Pathway	Cytokine Signaling in Immune system	BioSystems: REACTOME	2,97E-03	50	763
Pathway	Influenza A	BioSystems: KEGG	4,82E-03	19	173

Pathway	Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell	BioSystems: REACTOME	1,13E-02	16	136
Pathway	Osteoclast differentiation	BioSystems: KEGG	2,68E-02	15	130
Pathway	Herpes simplex infection	BioSystems: KEGG	4,47E-02	18	185

Table 3. Pathway enrichment analysis of a cluster of 593 DEGs in AAV versus healthy individuals (turquoise cluster on heatmap shown in Figure 4A.).

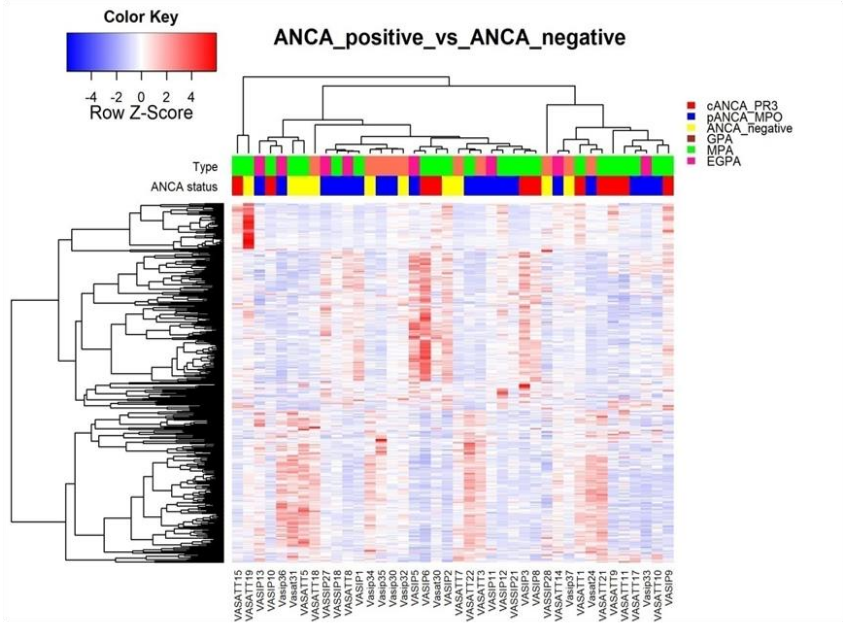
Category	Name	Source	p-value	Hit query list	Hit Genome
Pathway	Gene Expression	BioSystems: REACTOME	3,35E-06	79	1844
Pathway	rRNA processing	BioSystems: REACTOME	1,46E-04	19	203
Pathway	Eukaryotic Translation Elongation	BioSystems: REACTOME	2,67E-04	13	98
Pathway	rRNA processing in the nucleus and cytosol	BioSystems: REACTOME	3,31E-04	18	193
Pathway	Nonsense-Mediated Decay (NMD)	BioSystems: REACTOME	1,69E-02	12	121
Pathway	Ribosome	BioSystems: KEGG	4,24E-02	13	154

3.4 Differential Gene Signatures define ANCA positivity

Clinical and experimental data indicate the crucial role of ANCA in the pathogenesis of AAV, but whether ANCA positivity is accompanied by specific transcriptional signature remains elusive. By comparing the whole blood transcriptomic profile of ANCA (+) to ANCA (-) patients, we identified 516 DEGs $p<0.05$ and absolute $FC>0.58$ (Figure 5A). 295 DEGs were downregulated in ANCA (+) patients, whereas 221 were upregulated in the same group. PCA analysis showed that AAV patients were not clustered according to ANCA profile, which explains the small number of DEGs identfied (Figure 5B). The PPI network of the DEGs is shown in Figure 5C. Nodes with high degree connectivity were *CTNNB1* and *SPI1*. Enrichment analysis including all DEGs showed enriched pathways and processes such as pink/parkin mediated mitophagy, post-transcriptional modifications, mitochondrial transport system, intracellular metabolism, tranferrin/heme metabolism and Wnt signaling (Figure 5E). Immune related processes enrichment analysis including all DEGs, highlighted the importance of biological processes, such as IL signaling, leukocyte migration, immunoglobulin production and isotype switching, B cell mediated immunity, antigen processing and presentation, pattern recognition receptor signaling, erythorocyte and myeloid cell development, natural killer mediated immunity and T cell mediated cytotoxicity (Figure 5D).

Clustering analysis revealed two clusters of DEGs. The first cluster, composed of 219 DEGs, was enriched for mitochondrial cellular component and gene expression pathways (Table 4). The cluster of 297 DEGs associated only with hemoglobin metabolic processes (Table 5). These results highlighted the presence of transcriptional differences between ANCA (+) and ANCA (-) AAV patients, which are mainly related to mitochondrial components, pink/parkin mediated mitophagy, immunological processes and hemoglobin metabolism.

A



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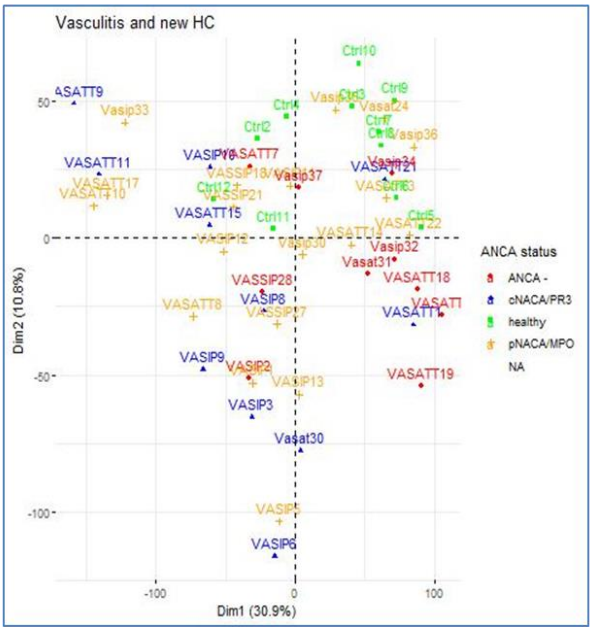

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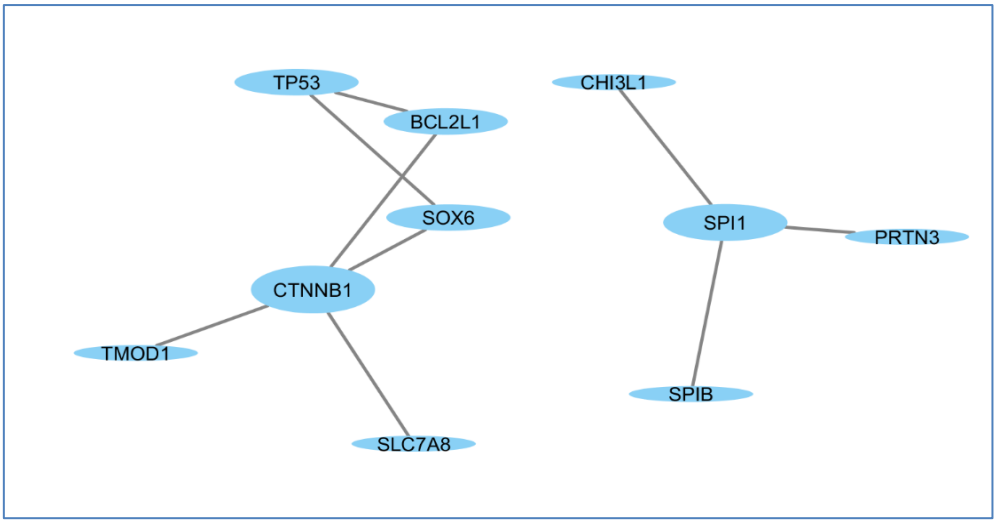
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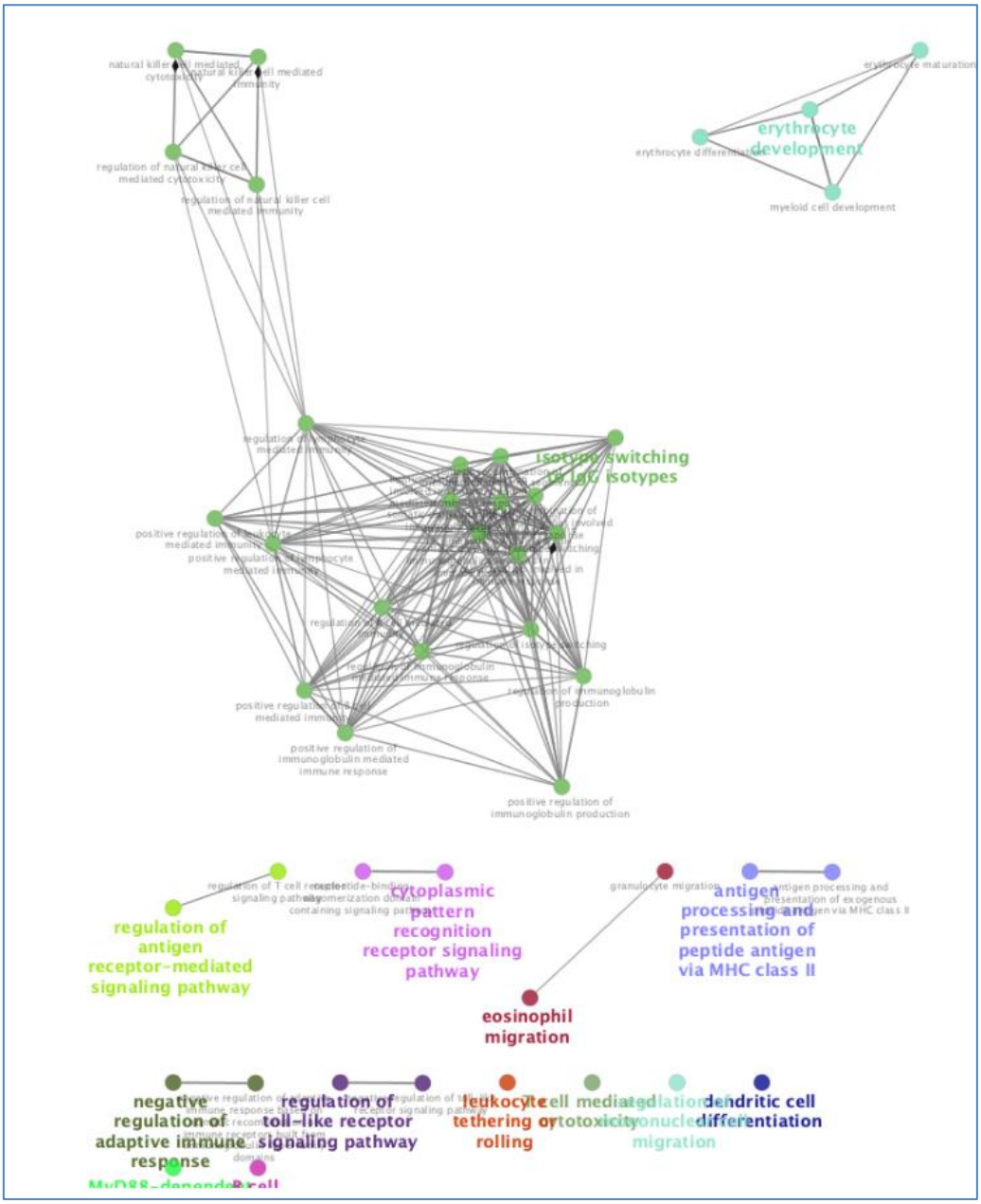
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myeloid cell development
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cytoplasmic pattern recognition receptor signaling pathway
antigen processing and presentation of peptide antigen via MHC class II
eosinophil migration
negative regulation of adaptive immune response
regulation of toll-like receptor signaling pathway
leukocyte tethering or rolling
dendritic cell differentiation

E

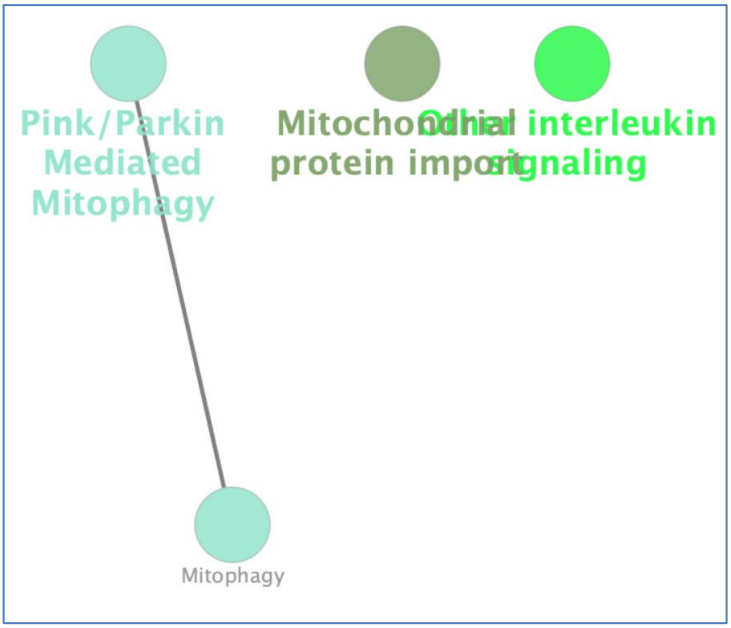


Figure 5. (A) Heatmap of DEGs in whole blood of ANCA(+) AAV patients versus ANCA(-) AAV patients. (B) PCA of whole transcriptome between ANCA(+) and ANCA (-) AAV. (C) PPI Network of identified DEGs in ANCA (+) versus ANCA (-) comparison. (D) Mechanistic map of biological processes with all DEGs (ANCA(+) AAV patients versus ANCA(-) AAV patients). (E)Pathway enrichment analysis with all DEGs (ANCA(+) AAV patients versus ANCA(-) AAV patients).

Table 4. Enrichment analysis of a cluster of 219 DEGs in ANCA(+) AAV patients versus ANCA(-) AAV patients.

Category	Name	Source	p-value	Hit query list	Hit Genome
GO: Cellular Component	mitochondrial part		4,46E-02	25	987
Pathway	Gene Expression	BioSystems: REACTOME	9,50E-05	43	1844

Table 5. Enrichment analysis of a cluster of 297 DEGs in ANCA(+) AAV patients versus ANCA(-) AAV patients.

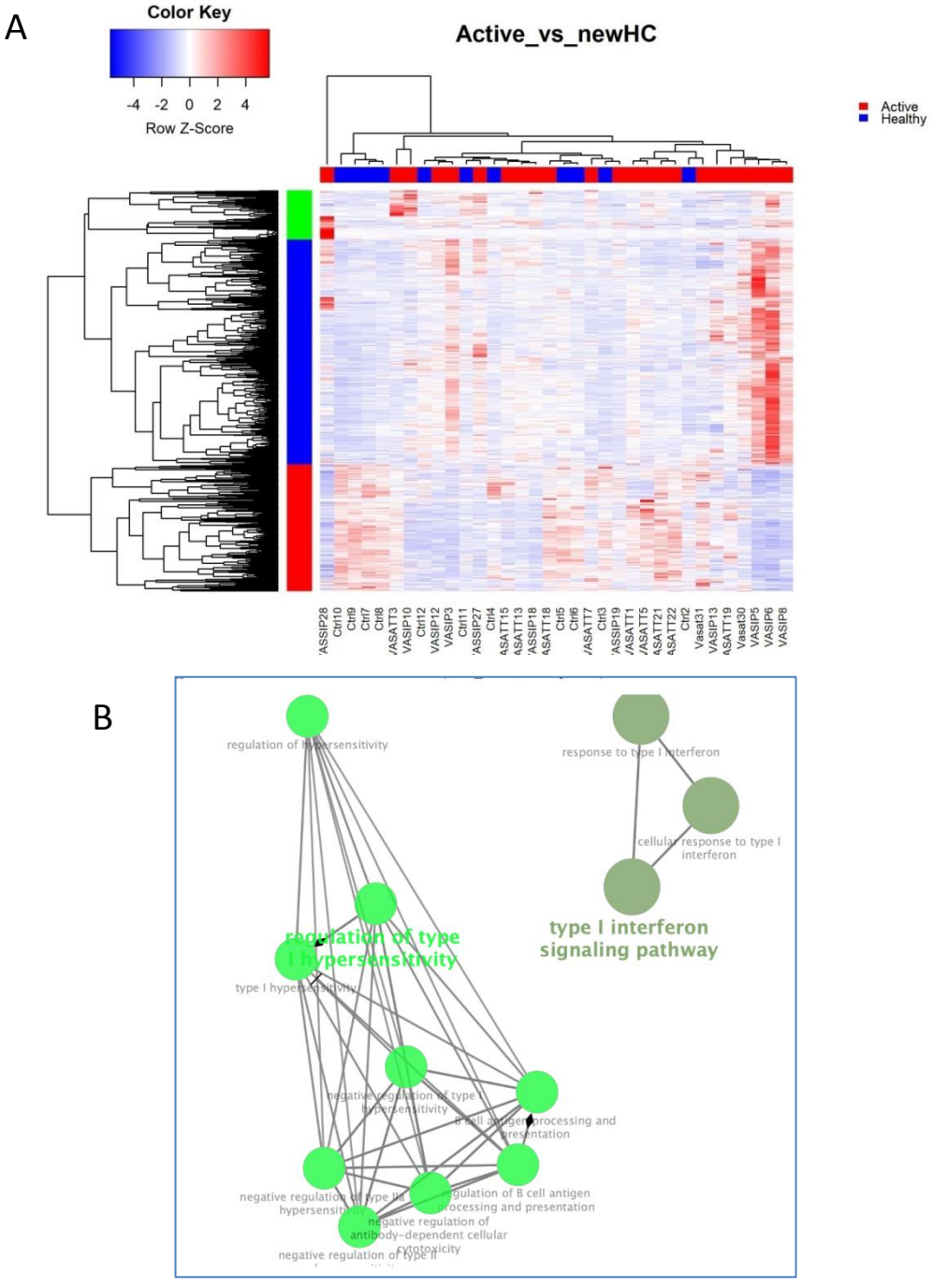
Category	Name	p-value	Hit query list	Hit Genome
GO: Biological Process	hemoglobin metabolic process	2,09E-03	5	19

3.5 Active Disease transcriptional landscape is broadly deregulated

Considering the extensive transcriptional perturbations in AAV patients, we asked to what extent activity status has an effect on the transcriptional profile of AAV. Whole blood transcriptome of patients with active disease at sampling was compared with healthy controls. The comparison revealed 941 DEGs ($p<0.05$ and absolute FC>1.5) (figure 6A). 645 genes were downregulated in active disease, whereas 296 genes were higher upregulated. Interestingly, many type 1 IFN associated genes, such as *USP18, IFIT1, IFIT3, OAS3, STAT2, IRF9, XAF1*, *DDX58* were downregulated in active AAV. Along the same lines, expression of genes related to neutrophil degranulation, such as *CPPED1, LPCAT1 CTSC, FCGR2A, CXCR1, GALNS, PTAFR, ADAM8, ALOX5, TSPAN14, DNASE1L1, FPR1* was lower in AAV cases compared to healthy controls. Targeted immulogical processes enrichment analysis of DEGs

suggested the involvement of type 1 IFN signaling and regulatory mechanisms underlying type 1 hypersensitivity reaction in active disease (Figure 6B). Pathway enrichment analysis revealed the enrichment of leukocyte differentiation, TNF suprefamily production and host defense mechanisms against pathogens (Figure 6C)

By hierarchical clustering of DEGs, 3 distinct gene clusters were identified. The first cluster included 298 genes, which were mainly enrinched in biological processes related to T cell receptor maturation, RNA processing, translation and peptide biosynthesis (table 6). The second cluster (115 genes) was enriched predominantly in type 1 IFN signaling, antiviral response (Table 7). The third cluster (528 genes) was mainly associated with neutrophil degranulation and innate immunity pathways (Table 8).



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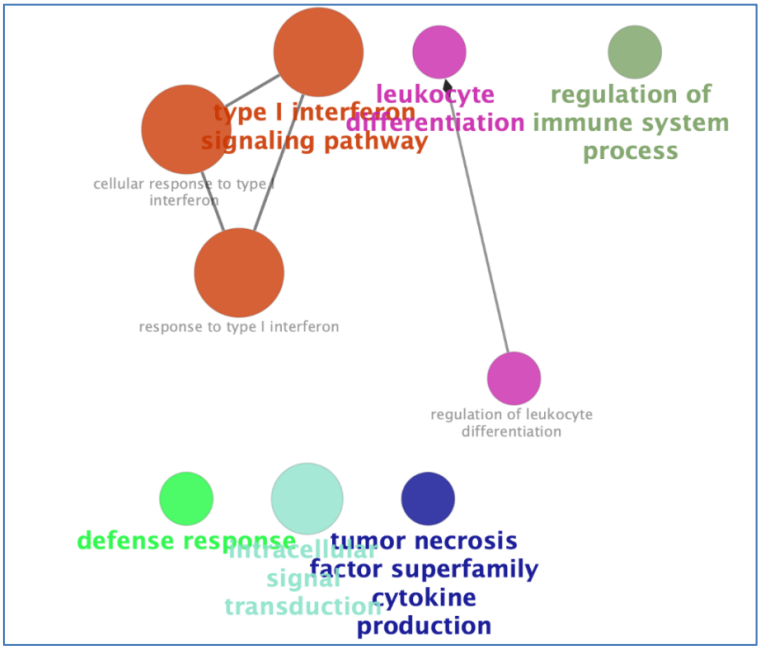


Figure 6. (A) Heatmap of DEGs in whole blood of AAV active disease patients versus healthy individuals. (B) Mechanistic map of immune regulation with all DEGs (Active versus healthy control). (C) Pathway enrichment analysis with all DEGs (Active versus healthy)

Table 6. Enrichment analysis of a cluster of 298 DEGs in active disease AAV patients versus healthy individuals.

Category	Name	p-value	Hit query list	Hit Genome
GO: Biological Process	somatic recombination of T cell receptor gene segments	2,52E-02	3	5
GO: Biological Process	T cell receptor V(D)J recombination	2,52E-02	3	5
GO: Biological Process	peptide biosynthetic process	3,53E-02	24	696
GO: Biological Process	translation	5,16E-02	23	667

Table 7. Enrichment analysis of a cluster of 115 DEGs in active disease AAV patients versus healthy individuals.

Category	Name	Source	p-value	Hit query list	Hit Genome
Pathway	Interferon alpha/beta signaling	BioSystems: REACTOME	6,64E-11	11	69
Pathway	Interferon Signaling	BioSystems: REACTOME	2,57E-09	14	202
Pathway	Cytokine Signaling in Immune system	BioSystems: REACTOME	6,62E-05	18	763
Pathway	Influenza A	BioSystems: KEGG	3,84E-03	8	173

Table 8. Enrichment analysis of a cluster of 528 DEGs in active disease AAV patients versus healthy individuals.

Category	Name	Source	p-value	Hit query list	Hit Genome
Pathway	Neutrophil degranulation	BioSystems: REACTOME	3,91E-06	35	492
Pathway	Innate Immune System	BioSystems: REACTOME	5,16E-04	59	1312
Pathway	Osteoclast differentiation	BioSystems: KEGG	2,04E-03	14	130

3.6 Transcriptional abnormalities accompany inactive disease status

Remission induction and maintenance is our therapeutic target in AAV, but whether this is reflected into transcriptional changes remains poorly understood. To determine a remission transcriptional signature, we compared the transcriptional profile of AAV patients in remission and healthy controls. 619 DEGs were identified $p<0.05$ and absolute FC>1.5 (figure 7A). 427 DEGs were dowregulated in remission and 182 were upregulated. Downregulation of type 1 interferon signaling and NLR mediated signaling genes, such as *SP18, OAS1, OAS2, OAS3, IRF7, IFIT1, IFIT3, STAT2* and *CARD17* suggested a "remission signature". Downregulation of genes associated with IFNγ signaling, such as *SOCS3, PTAFR, IRF9, FCGR1A, FCGR1B, PML* also characterized remission transcriptional profile. Noteworhty, genes linked to regulation of Wnt-mediated beta catenin signaling, such as *TCF7, CAMK4, AXIN2, LEF1, HDAC2* showed higher expression in remission compared to healthy control. Immune processes related enrichment analysis also showed enrichment of type 1 and IFNγ signaling pathways which would be potential therapeutic targets and restoration of their physiological function may be linked with remission status. It is of note, that neutrophil migration process was enriched, which further supports the role of neutrophils in disease pathogenesis (Figure 7B-C).

To detect gene subgroups, hierarchical clustering analysis was performed. Six clusters were identified but only 3 showed significant enrichment in processes/pathways.The first cluster (110 genes) was enriched in immune related pathways, such as IFN signaling, NLR mediated signaling and antiviral response (Table 9). The second cluster (121 genes) was significantly enriched in Wnt-mediated regulation of beta catenin and cancer pathways (Table 10). The fourth cluster (173 genes) was enriched in pathways related to adaptive immunity and interactions between lymphoid and non lymphoid cells (Table 11).

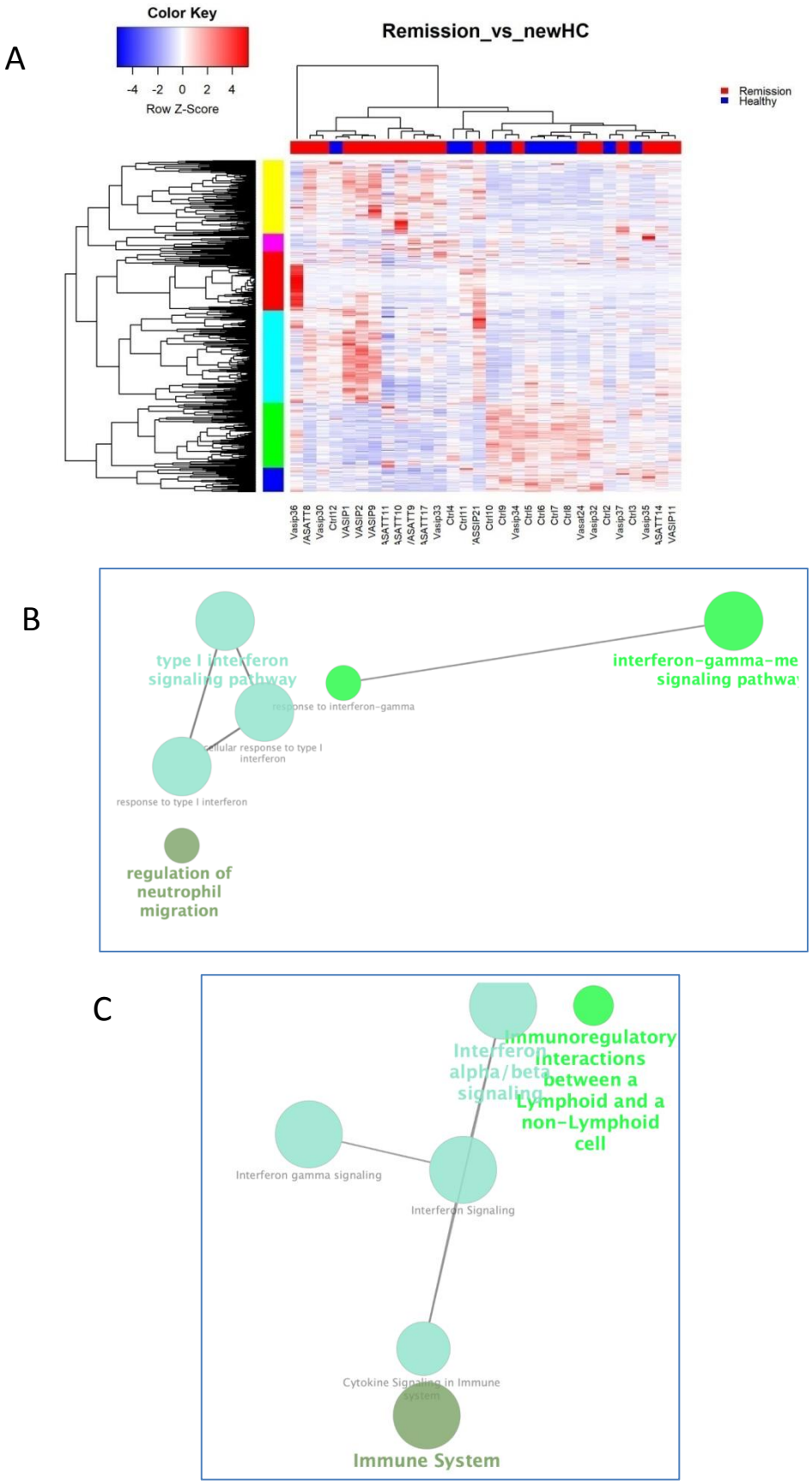


Figure 7. (A) Heatmap of DEGs in whole blood of AAV remission patients versus healthy individuals. (B) Mechanistic map of immune regulation with all DEGs (Remission versus healthy control). (C) Pathway enrichment analysis with all DEGs (Remission versus healthy control)

Table 9. Enrichment analysis of a cluster of 110 DEGs in remission AAV patients versus healthy (yellow cluster on heatmap shown in Figure 7A.).

Category	Name	Source	p-value	Hit query list	Hit Genome
Pathway	Interferon alpha/beta signaling	BioSystems: REACTOME	3,11E-19	16	69
Pathway	Interferon Signaling	BioSystems: REACTOME	2,44E-18	21	202
Pathway	Cytokine Signaling in Immune system	BioSystems: REACTOME	2,22E-10	25	763
Pathway	Interferon gamma signaling	BioSystems: REACTOME	2,74E-06	9	94
Pathway	Antiviral mechanism by IFN-stimulated genes	BioSystems: REACTOME	2,54E-04	7	77
Pathway	ISG15 antiviral mechanism	BioSystems: REACTOME	2,54E-04	7	77
Pathway	Influenza A	BioSystems: KEGG	5,58E-04	9	173
Pathway	Measles	BioSystems: KEGG	8,79E-04	8	134
Pathway	NOD-like receptor signaling pathway	BioSystems: KEGG	5,25E-03	8	170

Table 10. Enrichment analysis of a cluster of 121 DEGs in remission AAV patients versus healthy (red cluster on heatmap shown in Figure 7A.).

Category	Name	Source	p-value	Hit query list	Hit Genome
Pathway	Binding of TCF/LEF:CTNNB1 to target gene promoters	BioSystems: REACTOME	4,26E-03	3	7
Pathway	Regulation of Wnt-mediated beta catenin signaling and target gene transcription	BioSystems: Pathway Interaction Database	4,77E-02	5	79

Table 11. Enrichment analysis of a cluster of 173 DEGs in remission AAV patients versus healthy individuals (turquoise cluster on heatmap shown in Figure 7A.).

Category	Name	Source	p-value	Hit query list	Hit Genome
Pathway	Adaptive Immune System	BioSystems: REACTOME	3,21E-03	20	826

3.7 Identification of Active Disease transcriptional signature

Although active disease is associated with potentially life-threatening organ damage, accompanying whole blood transcriptional changes are not fully reported. We performed a differential expression analysis comparing AAV subjects with active disease to AAV patients in remission. We found 61 DEGs at a threshold pvalue<0.05 and log fold change (logFC)>0.58 (figure 6). 34 genes were downregulated, whereas 27 were upregulated. Active disease was

accompanied by lower expression of *IL18* gene, which typically stimulates IFNγ production by Th_1 cells.

According to the clustering analysis, we have shown two distinct gene clusters. The first cluster of DEGs (31 genes) was associated mostly with cytokine signaling events, especially those involving IL-4, IL-10, IL-23 or IL-13, cytoskeleton organization, hemoglobin metabolic processes (table 12), although statistical significance lacked. DEGs of the second cluster (30 genes) were not enriched in any pathway. Our analysis highlights the presence of a specific active-disease signature, which mainly is related with interleukin signaling events and cell motility.

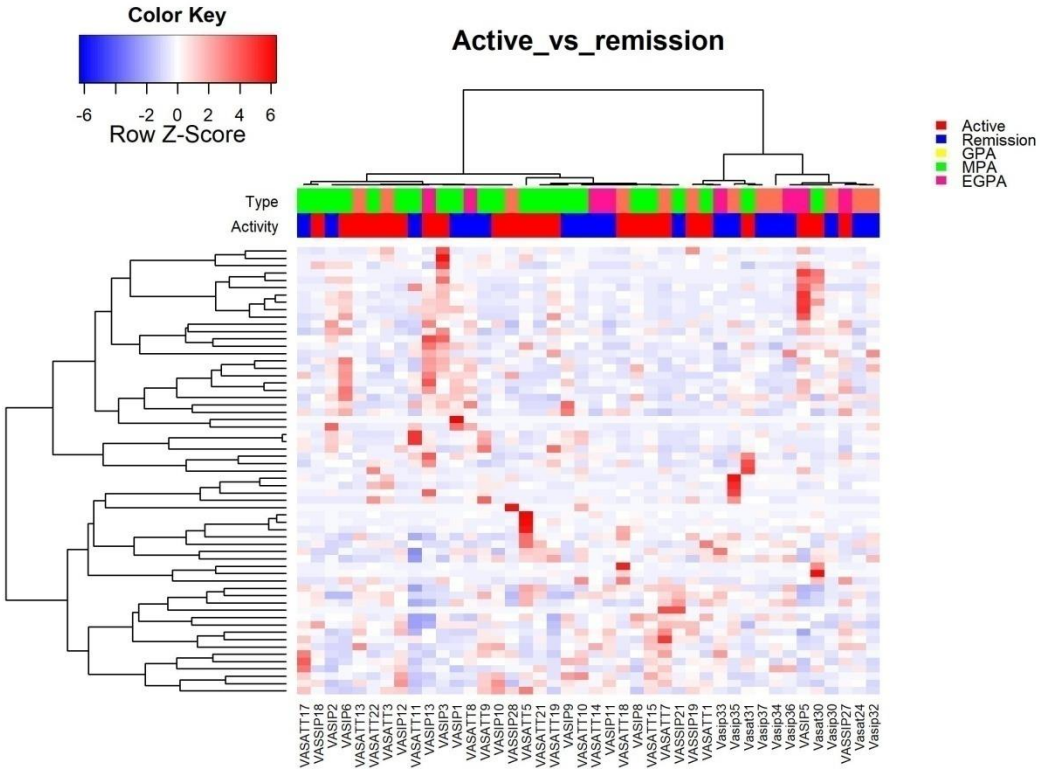


Figure 6. Heatmap of DEGs in whole blood of patients with active disease versus patients in remission.

Table 12. Enrichment analysis of a cluster of 31 DEGs in patients with active disease versus patients in remission

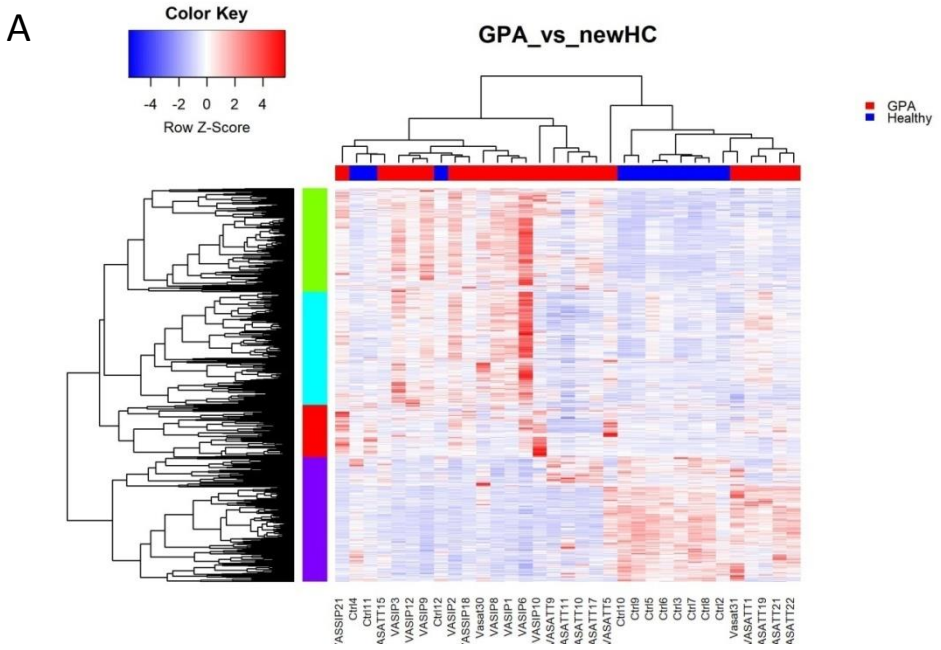
Category	Name	Source	p-value	Hit query list	Hit Genome
Pathway	African trypanosomiasis	BioSystems: KEGG	5,71E-03	3	35

3.8 Immune-related signatures differentiate GPA patients

Taking a step forward, we investigated whether specific gene signatures could efficiently differentiate AAV subtypes. To better understand the mechanisms of GPA, we compared GPA patients versus healthy controls. 1,225 DEGs ($p<0.05$ and absolute $FC>1.5$) identified

(Figure 7A). DEGs included 835 downregulated and 390 upregulated genes. PPI network constructed by RNEA is shown in Figure 7B. The degree of connectivity of certain nodes was strikingly higher than the average degree of other nodes in the network. Genes with high degree connectivity (hub genes) were *STAT1*, *LEF1*, *CEBPA*, *SPI1*, *CEBPE*, *CEBPD*, *STAT3*. Notably, hub genes are mainly associated with IFN signaling and myeloid differentiation. Interferon-related genes (*STAT1*, *IRF1*, *IRF9*, *IRF7*, *IFIT1*, *STAT2*, *STAT3, USP18),* innate immunity related genes (*TLR2*, *CXCL10*, *CXCL8, LCN2, CASP10, NOD1, TRAFD1*) and RIPK1-mediated necrosis genes (*RIK1, MLKL*) were downregulated in GPA patients compared to healthy controls. Additionally, genes of proteins involved in inflammasome complex formation and activation, such *as NLRC4, AIM2, CASP4, NLRP6* were downregulated in GPA cases compared to healthy controls. On the other hand, myeloid-differentiation related genes, such as *LEF1, GATA2, GATA3* were upregulated in GPA patients. Enrichment analysis of DEGs revealed that IFN, granulocyte activation and chemotaxis and regulation of innate and adaptive immunity response pathways were enriched, suggesting the critical role of these processes' deregulation in disease development (Figure 7C-D).

Clustering analysis defined 4 gene clusters associated mainly with immune signaling and cellular signal transduction. The gene set of the first cluster was enriched in type I and IFNγ signaling pathways, underscoring their major contribution in disease pathogenesis. Enrichment analysis of the same cluster additionally highlighted the potential pathogenetic involvement of cell death regulatory mechanisms, especially RIPK1-mediated necrosis in GPA (table 13). Genes defining the second cluster were statistically significantly enriched in neutrophil degranulation and innate immunity pathways (table 14). The gene set of the third cluster was involved in innate immunity responses, such as inflammasome complex formation, but also in caspase-8 and caspase-10 mediated NF-kB activation pathway (Table 15). Finally, the fourth cluster of genes, was mainly associated with processes involved in transcriptional and translational procedures and T cell differentiation (Table 16).



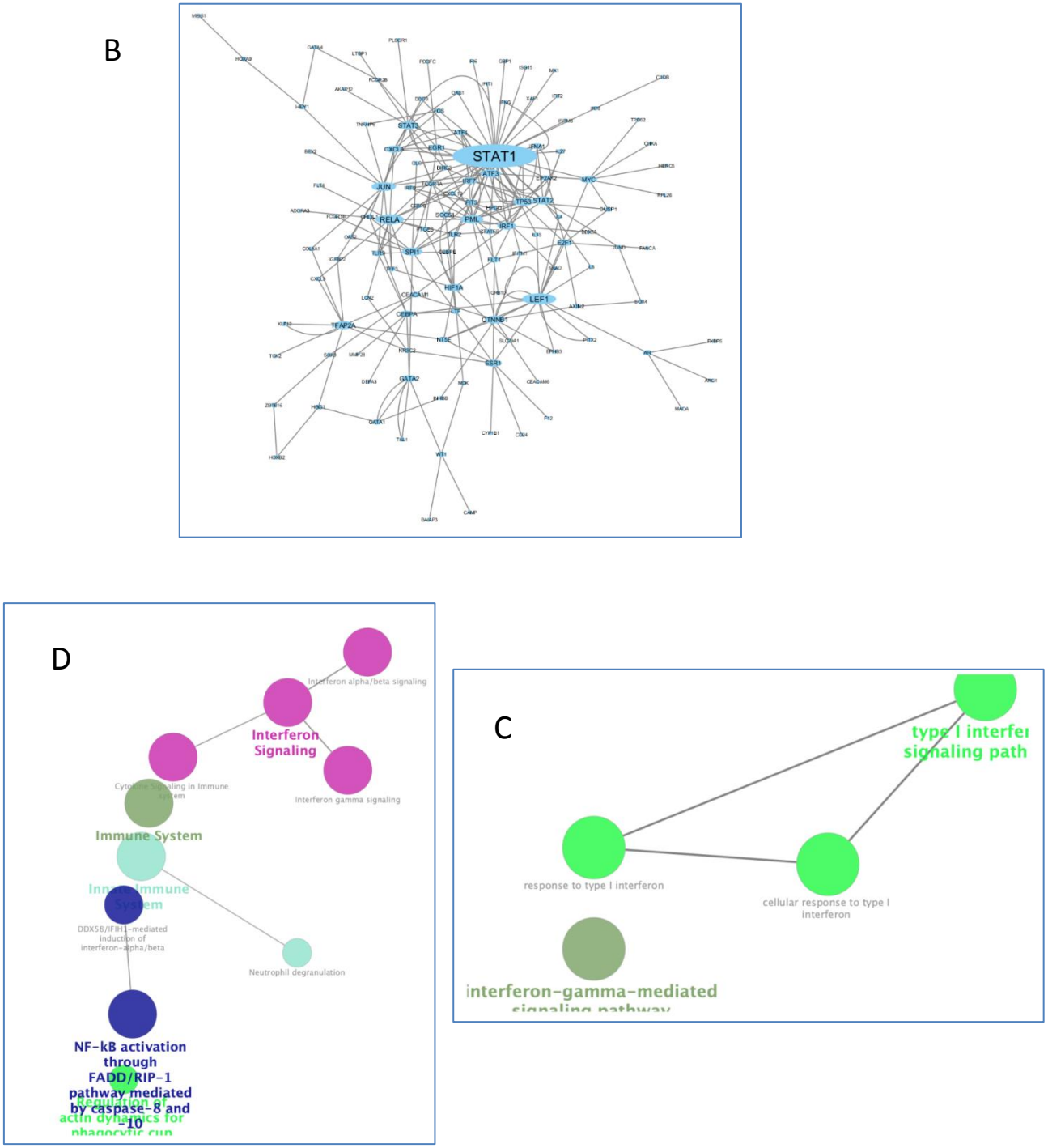


Figure 7. (A) Heatmap of DEGs in whole blood of patients with GPA versus healthy individuals. (B) PPI Network of identified DEGs in GPA versus healthy control comparison. (C)Mechanistic map of immune regulation with all DEGs (GPA versus healthy control). (D) Pathway enrichment analysis with all DEGs (GPA versus healthy control).

Table 13. Enrichment analysis of a cluster of 162 DEGs in patients with GPA versus healthy individuals.

Category	Name	Source	p-value	Hit query list	Hit Genome
Pathway	Interferon Signaling	BioSystems: REACTOME	6,10E-28	30	202

Pathway	Interferon alpha/beta signaling	BioSystems: REACTOME	1,18E-18	17	69
Pathway	Cytokine Signaling in Immune system	BioSystems: REACTOME	8,15E-15	34	763
Pathway	Interferon gamma signaling	BioSystems: REACTOME	1,20E-14	16	94
Pathway	Influenza A	BioSystems: KEGG	3,72E-09	15	173
Pathway	NOD-like receptor signaling pathway	BioSystems: KEGG	4,14E-08	14	170
Pathway	Hepatitis C	BioSystems: KEGG	4,73E-06	11	131
Pathway	Measles	BioSystems: KEGG	6,02E-06	11	134
Pathway	ISG15 antiviral mechanism	BioSystems: REACTOME	1,26E-04	8	77
Pathway	Antiviral mechanism by IFN-stimulated genes	BioSystems: REACTOME	1,26E-04	8	77
Pathway	Herpes simplex infection	BioSystems: KEGG	1,71E-04	11	185
Pathway	RIG-I/MDA5 mediated induction of IFN-alpha/beta pathways	BioSystems: REACTOME	2,51E-04	8	84

Table 14. Enrichment analysis of a cluster of 322 DEGs in patients with GPA versus healthy individuals.

Category	Name	Source	p-value	Hit query list	Hit Genome
Pathway	Neutrophil degranulation	BioSystems: REACTOME	7,51E-06	26	492
Pathway	Innate Immune System	BioSystems: REACTOME	1,31E-04	43	1312

Table 15. Enrichment analysis of a cluster of 353 DEGs in patients with GPA versus healthy individuals.

Category	Name	Source	p-value	Hit query list	Hit Genome
Pathway	NF-kB activation through FADD/RIP-1 pathway mediated by caspase-8 and -10	BioSystems: REACTOME	2,85E-02	4	13

Table 16. Enrichment analysis of a cluster of 388 DEGs in patients with GPA versus healthy individuals.

Category	Name	Source	p-value	Hit query list	Hit Genome
GO: Biological Process	ribonucleoprotein complex biogenesis		2,33E-05	28	468
GO: Biological	translational initiation		7,27E-05	17	194

Process					
GO: Biological Process	ribosome biogenesis		7,98E-05	22	322
GO: Biological Process	SRP-dependent cotranslational protein targeting to membrane		1,06E-04	12	94
GO: Biological Process	viral transcription		1,06E-04	16	176
GO: Biological Process	multi-organism metabolic process		1,86E-04	17	207
GO: Biological Process	rRNA processing		2,22E-04	19	260
GO: Biological Process	cotranslational protein targeting to membrane		2,36E-04	12	101
GO: Biological Process	viral gene expression		2,45E-04	16	187
GO: Biological Process	protein targeting to ER		2,63E-04	12	102
GO: Biological Process	rRNA metabolic process		3,34E-04	19	267
Pathway	Gene Expression	BioSystems: REACTOME	5,27E-09	71	1844
Pathway	GTP hydrolysis and joining of the 60S ribosomal subunit	BioSystems: REACTOME	1,63E-07	16	119
Pathway	Cap-dependent Translation Initiation	BioSystems: REACTOME	4,38E-07	16	127
Pathway	Eukaryotic Translation Initiation	BioSystems: REACTOME	4,38E-07	16	127
Pathway	L13a-mediated translational silencing of Ceruloplasmin expression	BioSystems: REACTOME	1,56E-06	15	119
Pathway	Translation	BioSystems: REACTOME	2,90E-06	17	165

3.9 Distinct transcriptional signatures define MPA

MPA is considered a distinct clinical phenotype of AAV, although difficulties in the differential diagnosis often arise. To partially elucidate MPA pathogenetic mechanisms, MPA cases were compared to healthy individuals. 1,093 DEGs ($p<0.05$ and absolute FC>1.5 (Figure 8A). 550 DEGs were downregulated and 543 DEGs were upregulated. The PPI regulatory constructed using the DEGs is shown in Figure 8B. Hub (nodes with degree connectivity) included *SP1, CEBPA, LEF1, JUN, MYC, CTNNB1, E2F1*. Interestingly, genes associated with neutrophil degranulation, such as *MMP8*, *CRISP3*, *TCN1*, *OLFM4*, *CEACAM6* and *CEACAM8* were upregulated in MPA compared to healthy controls. The gene encoding the granulocytic protein MPO, which is the major autoantigen targeted by ANCAs in MPA was upregulated in MPA samples (logFC= 2.65, p= 0.000179). Pathway enrichment analysis indicated the potential pathogenetic contribution of cell cycle, cellular motility but also p53 signaling pathways in MPA (Figure 8C-D).

Unsupervised hierarchical clustering analysis of DEGs identified 9 gene clusters. Genes included in the first cluster (272 genes) were mainly enriched in mitotic cell cycle pathways (Table 17). Enrichment analysis of the second gene set (294 genes) highlighted the deregulation of leukocyte motility and chemotaxis, neutrophil degranulation and innate immunity pathways in MPA (table 18). Biological processes associated with DNA damage response were identified by enrichment analysis of the third gene cluster, which consisted of 161 genes (Table 19). Moreover, upregulation of genes implicated in DNA damage response, such as *CENPJ*, *SPRED2*, *TRIAP1* and *ATM* suggest for the first time the implication of this process in MPA while this process was not found enriched in the analysis of other subtypes of AAV. Finally, the ninth cluster included 55 DEGs, which were enriched in cell cycle processes, p53 signaling pathways and further cell cycle checkpoint related pathways (Table 20).

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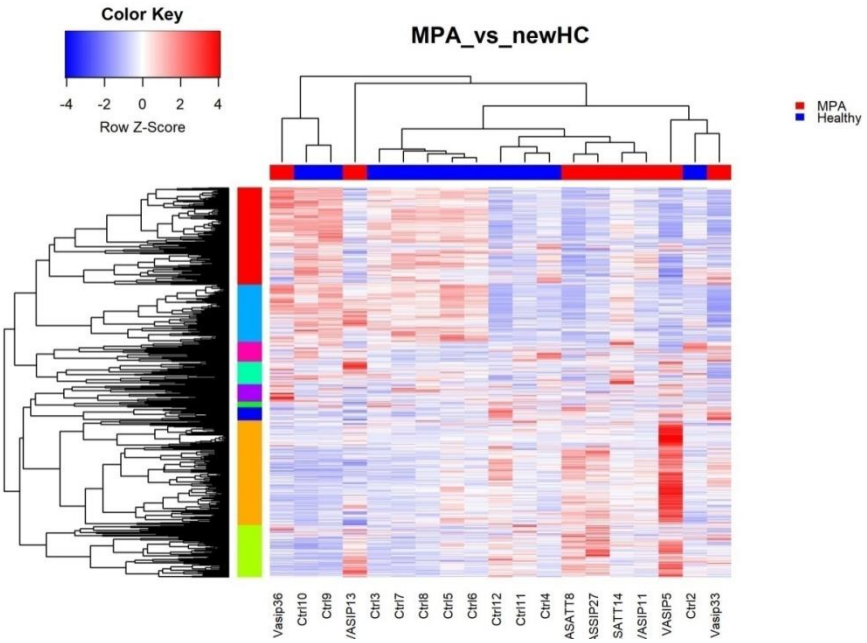

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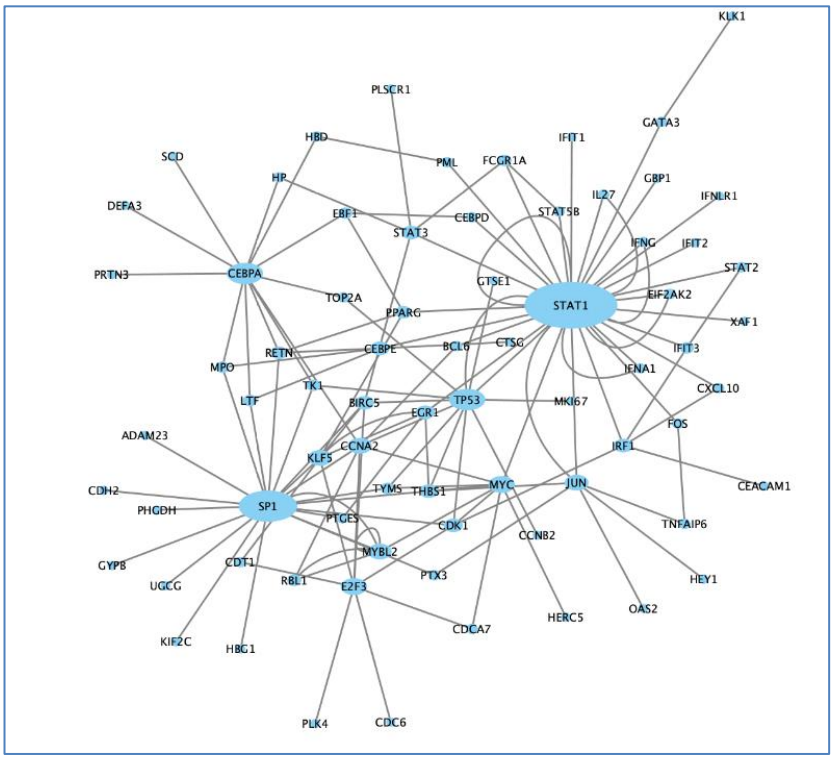


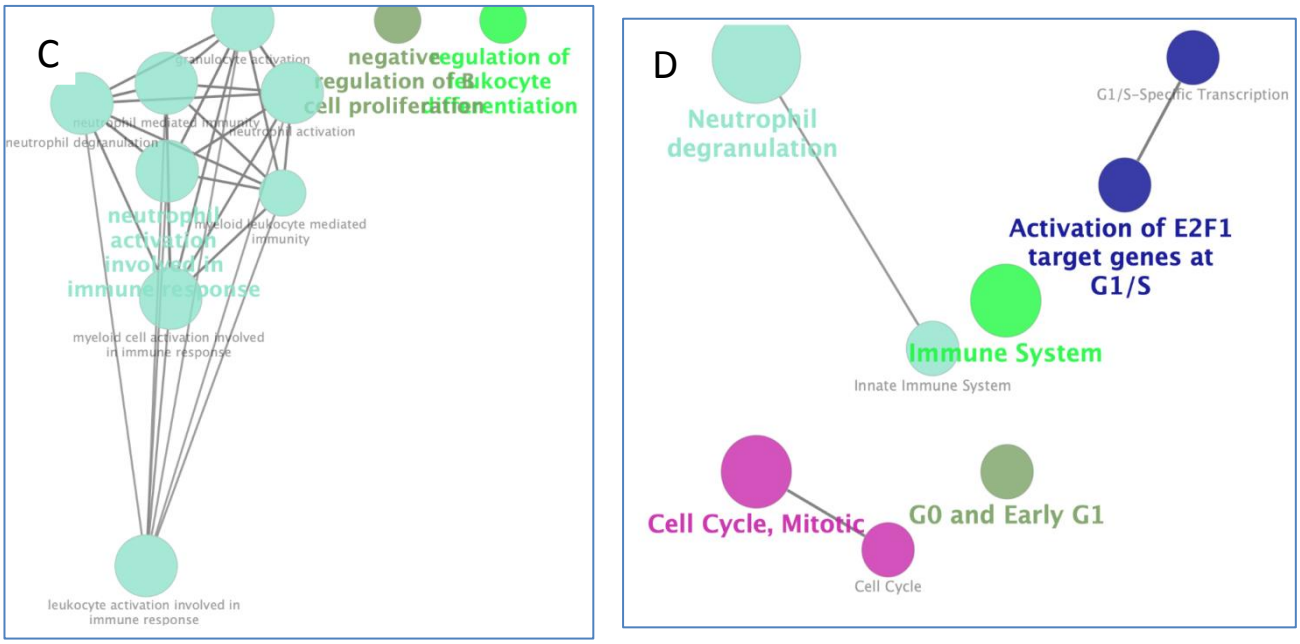


Figure 7. (A) Heatmap of DEGs in whole blood of patients with MPA versus healthy individuals. (B) PPI Network of identified DEGs in GPA versus healthy control comparison. (C)Mechanistic map of immune regulation with all DEGs (MPA versus healthy control). (D) Pathway enrichment analysis with all DEGs (MPA versus healthy control).

Table 17. Enrichment analysis of a cluster of 272 DEGs in patients with MPA versus healthy individuals.

Category	Name	Source	p-value	Hit query list	Hit Genome
Pathway	Cell Cycle, Mitotic	BioSystems: REACTOME	1,26E-05	25	517
Pathway	Mitotic G1-G1/S phases	BioSystems: REACTOME	4,96E-05	13	145
Pathway	Cell Cycle	BioSystems: REACTOME	1,26E-04	26	624
Pathway	G1/S Transition	BioSystems: REACTOME	4,80E-04	11	121
Pathway	E2F transcription factor network	BioSystems: Pathway Interaction Database	4,53E-03	8	73

Table 18. Enrichment analysis of a cluster of 294 DEGs in patients with MPA versus healthy individuals.

Category	Name	p-value	Hit query list	Hit Genome
GO: Biological Process	cellular extravasation	8,88E-03	7	52
GO: Biological Process	cell migration	9,21E-03	39	1300
GO: Biological Process	leukocyte migration	1,26E-02	18	386
GO: Biological Process	regulation of tumor necrosis factor superfamily cytokine production	1,39E-02	10	126
GO: Biological	tumor necrosis factor superfamily	1,71E-02	10	129

Process	cytokine production			
GO: Biological Process	localization of cell	3,40E-02	40	1428
GO: Biological Process	cell motility	3,40E-02	40	1428
GO: Biological Process	regulation of cellular extravasation	3,64E-02	5	27

Table 19. Enrichment analysis of a cluster of 161 DEGs in patients with MPA versus healthy individuals.

Category	Name	p-value	Hit query list	Hit Genome
GO: Biological Process	regulation of response to DNA damage stimulus	6,28E-05	11	156
GO: Biological Process	positive regulation of response to DNA damage stimulus	2,28E-02	6	70
GO: Biological Process	cellular response to DNA damage stimulus	2,88E-02	19	800
GO: Biological Process	DNA damage response, signal transduction by p53 class mediator	2,94E-02	7	108
GO: Biological Process	cellular macromolecule catabolic process	4,60E-02	21	977

Table 20. Enrichment analysis of a cluster of 55 DEGs in patients with MPA versus healthy individuals.

Category	Name	Source	p-value	Hit query list	Hit Genome
Pathway	Cell Cycle, Mitotic	BioSystems: REACTOME	3,60E-08	15	517
Pathway	Cell Cycle	BioSystems: REACTOME	4,19E-08	16	624
Pathway	Mitotic Prometaphase	BioSystems: REACTOME	7,07E-08	9	111
Pathway	Resolution of Sister Chromatid Cohesion	BioSystems: REACTOME	1,27E-06	8	103
Pathway	Aurora B signaling	BioSystems: Pathway Interaction Database	2,62E-06	6	39
Pathway	M Phase	BioSystems: REACTOME	5,85E-04	9	311
Pathway	Separation of Sister Chromatids	BioSystems: REACTOME	1,19E-03	7	170
Pathway	Mitotic Anaphase	BioSystems: REACTOME	1,81E-03	7	181
Pathway	Mitotic Metaphase and Anaphase	BioSystems: REACTOME	1,88E-03	7	182

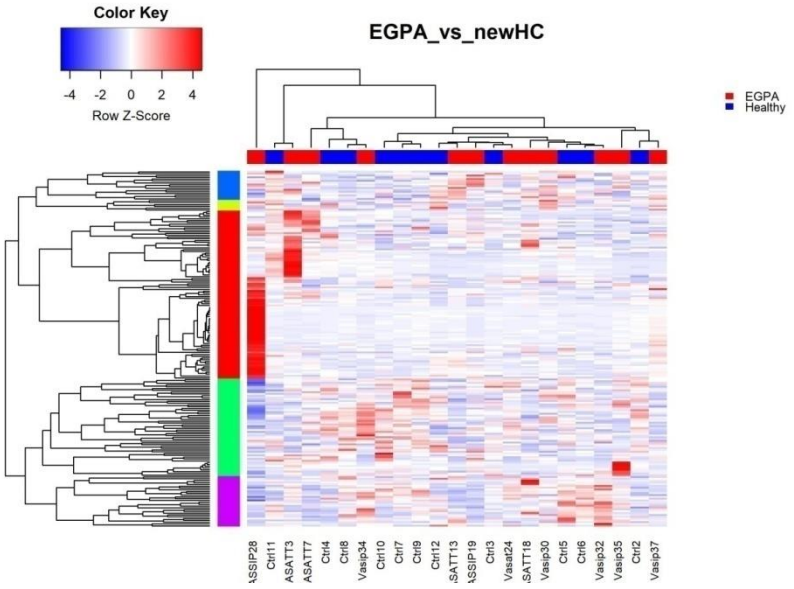
3.10 Differential Gene signatures define EGPA

EGPA is a complicated clinical entity, which often is accompanied by atopic manifestations. The biology that governs EGPA is poorly understood and data regarding peripheral blood transcriptional profile of EGPA patients are limited. To partially clarify disease's pathogenesis, we performed a whole blood differential expression analysis comparing EGPA to healthy individuals. We identified 197 DEGs (pvalue<0.05 and log fold change (logFC)>0.58) (figure 8A). 128 genes were downregulated in EGPA, whereas 69 genes were upregulated. Interestingly, lower expression of *IL-5RA*, which is associated with resistant

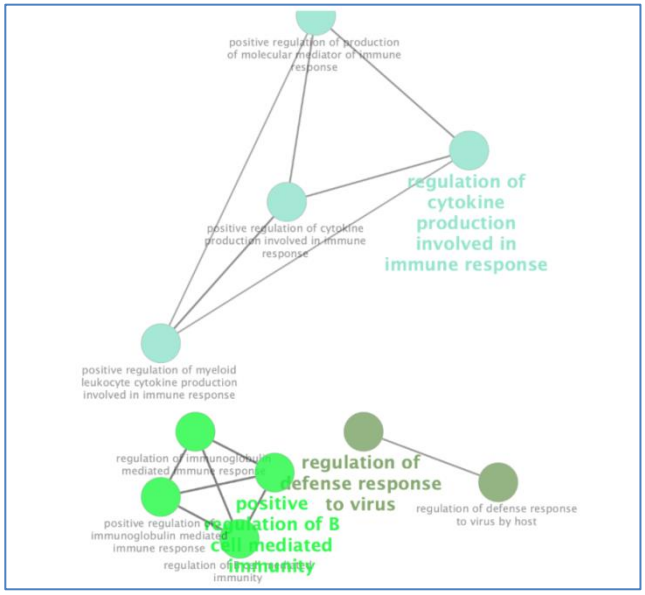
allerigic asthma was identified in EGPA patients. On the other hand, upregulation of genes associated with VEGF-VEGFR signaling, such as *ANGPT1*, *SPRED2*, *ELMO2* characterized EGPA transcriptome. Pathway enrichment analysis with all DEGs revealed deregulation of type I IFN signaling and abnormalities of B cell regulation pathways (figure 8B-D).

Hierarchical clustering of DEGs has shown 5 distinct gene clusters. The first cluster included 93 genes, which were mainly enriched in type I IFN, cytokine signaling and response to virus related pathways (table 21). Processes associated with extracellular polysaccharide biosynthesis were enriched in the 6 genes, which formed second gene cluster, although statistically significant enrichment was not reached. 28 genes of the third gene set were related to VEGF signaling and intracellular signaling (MAPK1/MAPK3, RAS) pathways, however statistical significance lacked. Interestinlgy, a fourth cluster (16 genes) included genes which may play a role in biological processes associated with CNS cell migration and motilty.

A



B



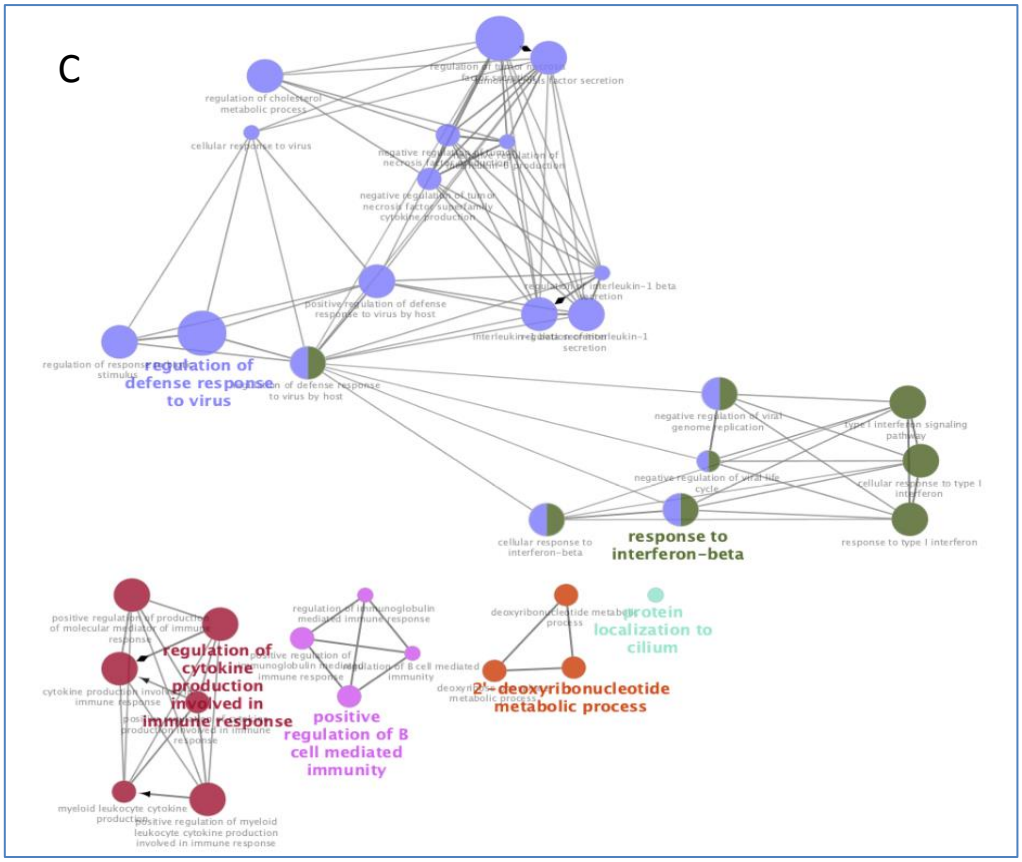


Figure 8. (A) Heatmap of DEGs in whole blood of patients with EGPA versus healthy individuals. (B)Mechanistic map of biological regulation with all DEGs (EGPA versus healthy control). (C) Mechanistic map of biological regulation regulation with all DEGs (EGPA versus healthy) (D) Pathway enrichment analysis with all DEGs (MPA versus healthy control).

Table 21. Enrichment analysis of a cluster of 93 DEGs in patients with EGPA versus healthy individuals.

Category	Name	p-value	Hit query list	Hit Genome
GO: Biological Process	defense response to virus	2,32E-03	10	343
GO: Biological Process	response to virus	2,37E-03	11	428
GO: Biological Process	immune response	9,34E-03	20	1572

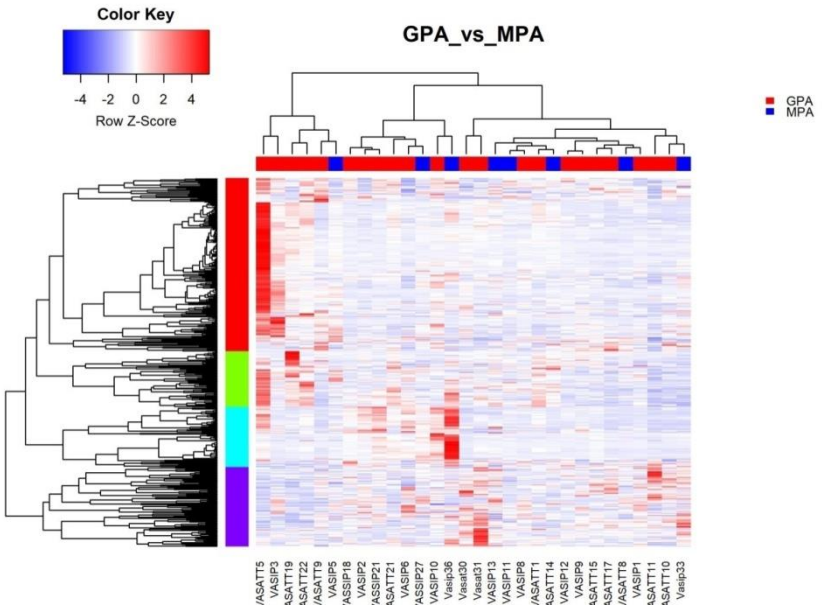
GO: Biological Process	defense response	1,92E-02	20	1651
GO: Biological Process	type I interferon signaling pathway	2,72E-02	5	81
GO: Biological Process	cellular response to type I interferon	2,88E-02	5	82
GO: Biological Process	regulation of immune effector process	3,35E-02	10	466

3.11 Interferon signature differentiates GPA from MPA

GPA and MPA are heterogenous clinical and histological entities with many overlapping features. To explore whether clinical variability is also reflected at the transcriptomic level, a comparison between GPA (n=22 samples) and MPA (n=8) patients was performed. 398 DEGs were identified ($p<0.05$ and absolute FC>0.58) (figure 9A). Specifically, 302 DEGs were downregulated in GPA samples and 96 were upregulated in GPA samples. To acquire a deeper understanding regarding biological processes and pathways that differentiate GPA from MPA, PPI network was constructed. *STAT1, CEBPA, SP1, E2F3, JUN* and *TP53* were identified as hub genes (Figure 9B). Genes related to type 1 IFN or IFNγ signaling, such as *OAS2*, *OAS3*, *XAF1*, *IFIT2*, *IFIT1*, *IFIT3*, *STAT1*, *STAT2, FCGR1A* were upregulated in MPA patients compared to GPA subjects. Transcriptional alterations of neutrophil activation and degranulation associated genes were identified. Upregulation of neutrophil granule protein genes, such as *AZU1*, *CEACAM8*, *MPO*, *LCN2*, *OLR1*, *SLC2A5*, *CEACAM1*, *DEFA4*, *RNASE3* differentiated MPA from GPA samples. Additionally, enrichment analysis of DEGs highlighted perturbations of specific biological processes and pathways that differentiate GPA from MPA. These processes included granulocyte differentiation and chemotaxis, type I IFN, IFNγ signaling, pattern recognition receptor signaling, T cell co-stimulation, antigene processing, macrophages activation, cellular and humoral response to microbes and viruses (Figure 9C). Pathway enrichment analysis, confirmed differences regarding type I IFN signaling, granulocyte degranulation and maturation, and additionally demonstrated novel pathways such as cell cycle, cell cycle checkpoints, intracellular metabolism, platelet function, megakaryocyte development, extracellular matrix biosynthesis and p53 signaling regulation (Figure 9D).

In order to identify gene clusters of pathogenetic importance, hierarchical clustering of DEGs was performed. Enrichment analysis of the first cluster (187 genes), demonstated involvement of pathways mainly related to cell cycle, cell cycle checkpoints, innate immunity, p53 signaling and purine biosynthesis (Table 22). The second cluster included 65 genes, which were mostly enriched in type I IFN and IFNγ signaling, pattern recognition receptor signaling and cytokine signal transduction pathways (Table 23).

A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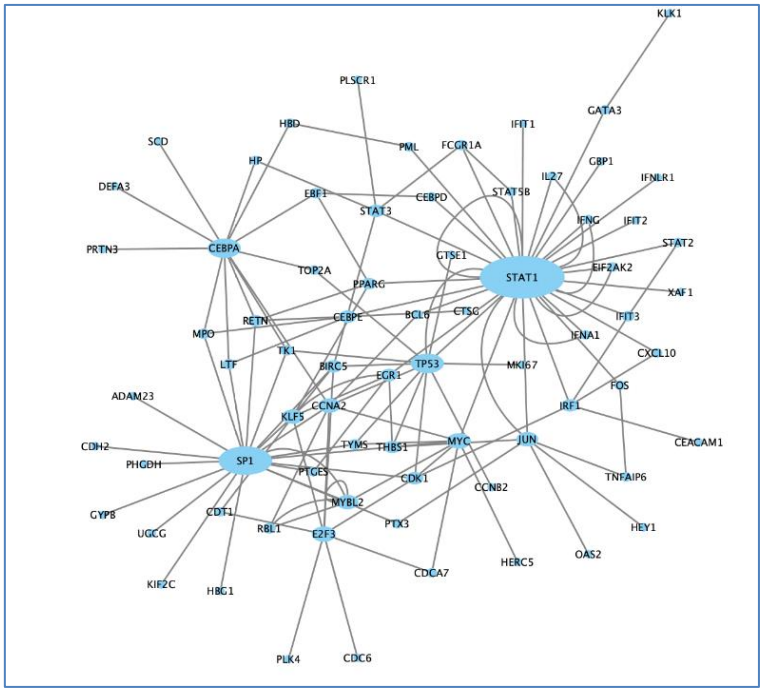
Row Z-Score
GPA_vs_MPA
GPA
MPA

B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PLSCR1
GATA3
HBD
IFIT1
SCD
PML
FCGR1A
HP
GBP1
IL27
IFNLR1
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EBF1
CEBPD
STAT5B
STAT3
IFNG
IFIT2
PRTN3
CEBPA
STAT2
GTSE1
TOP2A
STAT1
EIF2AK2
PPARG
XAF1
RETN
CEBPE
BCL6
CTSG
IFIT3
MPO
IFNA1
TK1
CXCL10
LTF
BIRC5
TP53
MKI67
EGR1
FOS
ADAM23
CCNA2
IRF1
KLF5
CDH2
TYMS
THBS1
MYC
JUN
CEACAM1
PHGDH
SP1
PTGES
CDK1
TNFAIP6
CCNB2
GYPB
CDT1
MYBL2
UGCG
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OAS2
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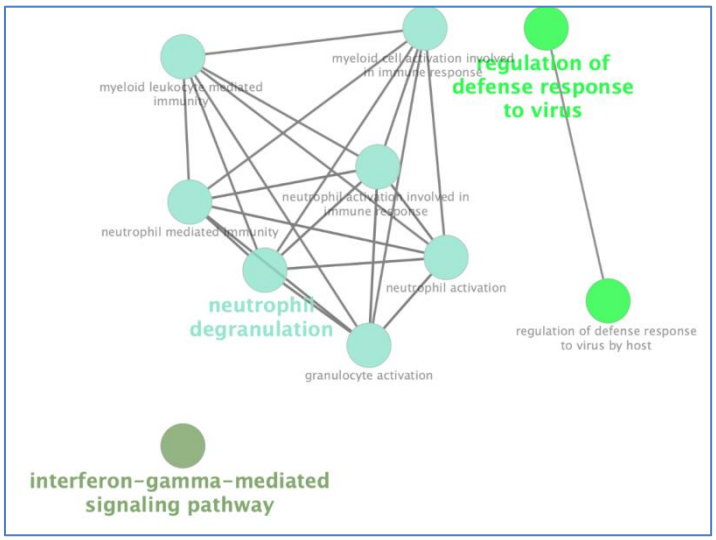
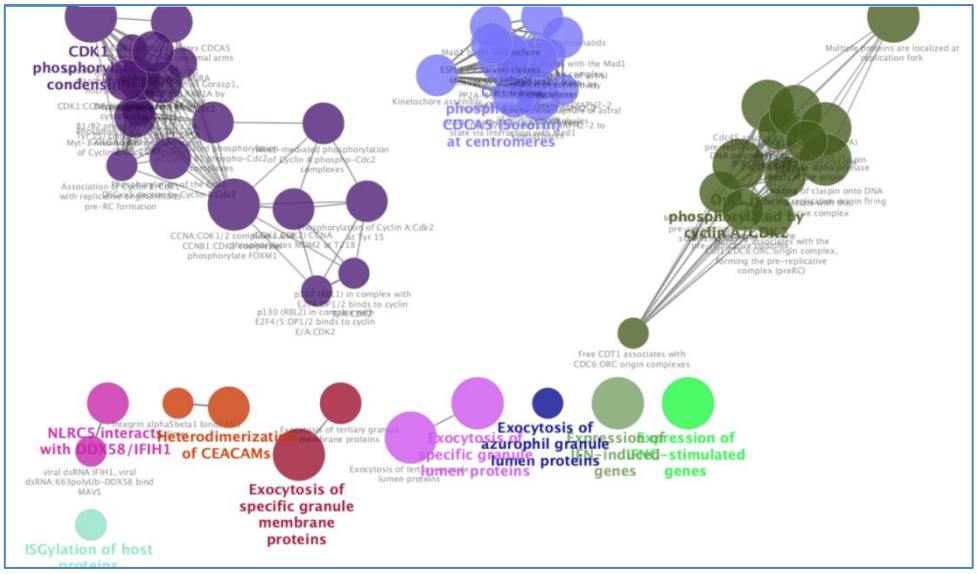
PLK4
CDC6

C


myeloid leukocyte mediated immunity
myeloid cell activation involved in immune response
regulation of defense response to virus
neutrophil activation involved in immune response
neutrophil mediated immunity
neutrophil degranulation
neutrophil activation
granulocyte activation
regulation of defense response to virus by host
interferon-gamma-mediated signaling pathway

D



Εικόνα 1Figure 8. (A) Heatmap of DEGs in whole blood of patients with GPA versus MPA. (B) PPI Network of identified DEGs in GPA versus MPA comparison. (C) Mechanistic map of immune regulation with all DEGs (GPA versus MPA). (D) Pathway enrichment analysis with all DEGs (GPA versus MPA).

Table 22. Enrichment analysis of a cluster of 187 DEGs in patients with GPA versus MPA.

Category	Name	Source	p-value	Hit query list	Hit Genome
Pathway	Cell Cycle	BioSystems: REACTOME	5,54E-20	44	624
Pathway	Neutrophil degranulation	BioSystems: REACTOME	2,62E-15	35	492
Pathway	Mitotic G1-G1/S phases	BioSystems: REACTOME	3,27E-13	20	145
Pathway	E2F mediated regulation of DNA replication	BioSystems: REACTOME	8,95E-11	11	34
Pathway	S Phase	BioSystems: REACTOME	3,52E-08	15	132
Pathway	Mitotic Prometaphase	BioSystems: REACTOME	3,94E-08	14	111
Pathway	Resolution of Sister Chromatid Cohesion	BioSystems: REACTOME	2,01E-07	13	103

Table 23. Enrichment analysis of a cluster of 65 DEGs in patients with GPA versus MPA.

Category	Name	Source	p-value	Hit query list	Hit Genome
Pathway	Interferon Signaling	BioSystems: REACTOME	2,01E-18	18	202
Pathway	Interferon gamma signaling	BioSystems: REACTOME	7,41E-10	10	94
Pathway	Interferon alpha/beta signaling	BioSystems: REACTOME	1,75E-09	9	69
Pathway	Cytokine Signaling in Immune	BioSystems:	2,41E-09	19	763

	system	REACTOME			
Pathway	Influenza A	BioSystems: KEGG	1,37E-04	8	173
Pathway	NOD-like receptor signaling pathway	BioSystems: KEGG	1,96E-03	7	170
Pathway	Herpes simplex infection	BioSystems: KEGG	3,44E-03	7	185
Pathway	Hepatitis C	BioSystems: KEGG	6,15E-03	6	131
Pathway	Measles	BioSystems: KEGG	7,01E-03	6	134

3.12 Whole blood transcriptome discriminated AAV active renal disease versus active Lupus nephritis (LN)

AAV renal involvement dramatically deteriorates patients' prognosis, whereas AAV glomerulonephritis is characterized by specific histopathological findings, such pauci immunity. On the other hand, LN also is considered potentially life-threatening manifestation of SLE, which often is accompanied by extensive immune complex depositions in glomeruli. Whether differences between these clinical entities are also mirrored by peripheral blood transcriptome remains unknown. To assess, whether possible similarities or differencies between active AAV renal disease and active LN are present, we performed PCA of blood gene expression between 12 AAV patients with active renal disease and 32 active LN patients. PCA differentiated clearly the two groups, although technical issues and clinical heterogenecity regarding confounding factors might influence the result (figure 11).

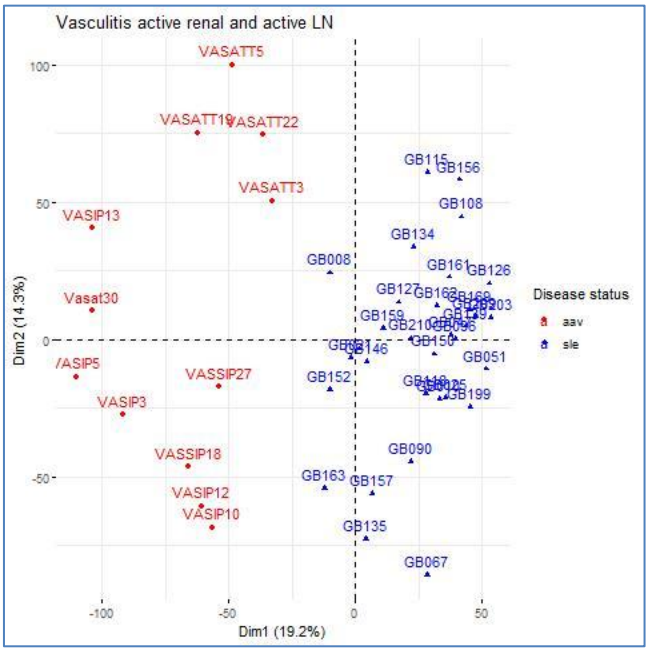


Figure 5. PCA of whole transcriptome between active AAV renal disease and active LN.

Discussion

The presented findings reveal novel insights into pathogenetic transcriptional deregulations underlying AAV. This study provides an extensive characterization of gene signatures in AAV associated with disease activity, subtype and ANCA positivity. It comprenhensively details the differentially expressed genes and related biological processes and signaling pathways that correspond to AAV phenotype, specific subtypes, activity status and ANCA profile. Finally, these results indicate the presence of important transcriptional differences between AAV renal disease and LN.

The transcriptomic profiling of AAV imply extensive deregulation of type 1 and IFNγ signaling pathways. In contrast to previous studies [143, 144], which did not detect any IFN signature, this suggest a downregulation of genes related to type 1 and IFNγ related pathways in AAV. The pathogenetic role of type 1 IFN in rheumatic diseases is well established [154]. IFNα is predominantly produced by plasmatocytoid DC (pDC), whereas several cell types (fibroblasts, epithelial cells, dendritic cells, phagocytes and synoviocytes) secrete IFNβ. Moreover, constituve expression of IRF7 by pDC, enables them to promptly generate high levels of IFNα, without previous "priming". Recognition of microbial peptides or neucleic acids by surface or cytosolic PPRs induces type 1 IFN production. Notably, STING, the stimulator of interferon genes is a cytosolic nucleic acid sensor, which stimulates type 1 IFN upon detection of nucleic acids in the cytoplasm. However, canonical and non-canonical inflammasome activation results in cascpase-1 mediated degradation of cyclic GMP-AMP synthase (cGAS), which is necessary for STING activation and type 1 IFN production. IFNβ production is induced through TNF receptors (TNFRs), such as receptor activator of nuclear factor-κB (RANK) and TNFR2. Interestingly, in phagocyte and endothelial cells, TNF can trigger an IFNβ autocrine loop, which in synergy with canonical TNF signal stimulates sustainable transcription 1 of inflammatory genes and "delayed" expression of STAT1-dependent interferon stimulated genes (ISGs). In human endothelial cells, TNFR2 engagement gives rise to this cascade, which in turn facilitates monocyte chemotaxis. Immodarate inflammasome mediated restriction of STING induced type 1 IFN production provides a possible interpretetation of the downregulation of IFN-related genes in AAV, although our results do not suggest an upregulation of inflammasome complex pathways. Additionally, corticosteroids remain a cornerstone of AAV treatment, whereas suppressive effects of corticosteroids on human immunome are known [155]. In this study, sampling timepoint often closely preceded high dose corticosteroid regimes, which might partially explain this result.

On the other hand, several suppressive mechanisms avert excessive type 1 IFN responses and ensure homeostasis [156]. Briefly, internalization of IFNα receptor (IFNAR), augmented expression of negative regulators (such as SOCS or USP18) or induction of regulatory non-coding RNAs counterbalance type 1 IFN pathway activation. In this analysis, type 1 IFN induced downregulation of SOCS1 and USP18 in AAV patients compared to healthy controls might suggest a suppression of type 1 INF signaling, although the downregulation of type 1 IFN pathway can not be explained. High avidity cross-linking of ITAM-associated receptors, pro-inflammatory signaling pathways, such as those activated by IL-1, TLRs, oxidative or metabolic stress limit cellular IFN responsiveness though internalization of IFNAR.

Interestingly, an inhibitory p38-mediated mechanism promotes IFNAR ubiquitylation and degradation and normally plays a paramount role in controlling the magnitude of type 1 IFN-eliciting effects [157, 158]. miRNAs can control gene expression at post-transcriptional level. PPR signaling is linked with high expression of miR-155, which in turn suppresses type 1 IFN signaling in CD8+ T cells [159, 160, 161]. Defects in these negative regulatory mechanisms might contribute to transcriptomic disbalance seen in AAV, but further experiments are required to validate such a finding.

Epigenetic modifications exert a regulatory function in many autoimmune diseases. In unstimulated cells, transcription factor FOXO3 inhibits interferon-stimulated gene (ISG) transcription, whereas high nucleosome occupancy due to low abundance of CpG islands in ISG promoters inhibits ISG transcription [156]. Upon type 1 IFN stimulation, binding of IFN-stimulated gene factor 3 (ISGF3) (composed by STAT1, STAT2 and IRF9) to ISG promoters and enhancers results in recruitment of chromatin remodeling complexes, which in turn makes specific regulatory elements (promoters and enhancers) accessible to transcriptional machinery. Moreover, demethylation of histone H3 at lysine 9 (H3K9me2) serves as a suppression of ISG expression in a cell-type specific manner [162]. Alterations of the chromatin regulatory landscape affecting the mechanisms reported above might be relevant to AAV and suggest a possible clarification of the observed type 1 IFN downregulation.

CD8 T cell exhaustion is associated with better clinical outcome in AAV [163]. In short, CD8 T cell exhaustion is defined by upregulation of inhibitory receptors, such as PD-1 or LAG-3 and downregulation of memory markers, such as IL7R [163, 164]. CD8 T cell exhaustion is facilitated through persistent antigen stimulation and absence of accessory costimulation [163]. Additionally, suppression of IFNγ production has been documented as a consequence of CD8 T cell exhaustion [164]. Although *IL7R* expression is elevated in AAV patients compared to healthy controls and the presence of CD8 T cell exhaustion signature has not been confirmed, it can be hypothesized that downregulation of IFNγ pathway in AAV could possibly be linked with T cell exhaustion. A specific exhaustion signature might not be apparent due to confounding influence of expression of other cell types included in whole blood samples.

Neutrophils activation and degranulation play a determinant role in the pathogenesis of AAV. ANCA activated neutrophils generate ROS, release destructive enzymes and extrude NETs at the site of inflammation [99, 100, 111]. In contrast to previously reported data, there were no expression differences of neutrophil granule protein genes and genes involved in neutrophil degranulation pathway were downregulated in AAV. In the first place, this finding does not underscore the critical role of neutrophils in AAV pathogenesis. However, extravasation of ANCA-activated neutrophils might not allow the identification of an upregulated granulocytic specific signature the analysis. Moreover, corticosteroid therapy exerts a direct suppressive effect on granulocytic activity and might be implicated in my results. Finally, whole blood derived gene expression signatures often lack required specifity to identify cell type targeted signatures.

Clinical and experimental data support the notion that ANCA are pathogenetic, although their prognostic value is controversial [91-96]. In my results, ANCA positivity is accompanied

by deregulation of pathways related to mitophagy, haemoglobin metabolism, immunoglobulin isotype switching, PPRs signaling and mitochondrial cellular compartment. The role of epitope spreading in conversion of natural ANCAs into pathogenetic autoantibodies has been previously reported [83]. My findings further emphasise the implication of biological features of humoral immunity, such as class switching in the pathophysiology of ANCA (+) AAV. Despite the fact that gene expression differences are overt, should also be taken into account that conventional serologic assays often fail to detect ANCAs and some AAV patients may falsely have been classified as ANCA (-) AAV [83].

My analysis provides unique insights regarding genetic mechanisms in AAV patients with active disease. Type 1 IFN signaling and meutrophil degranulation pathways represent two robust signals that characterize active disease status. Intriguingly, patients with active disease exhibited a downregulation of these pathways. Corticosteroid treatment prior to sampling, demargination of circulating neutrophils and mobilization of immature neutrophils from bone marrow appear to be potential confounder of gene expression. A “remission signature” enriched mainly in type 1 IFN, IFNγ and Wnt-mediated beta catenin signaling genes was also identified. Specifically, lower expression of immune related type 1 IFN and IFNγ pathways along with upregulation of Wnt-mediated regulation of beta catenin suggested an inactive status signature. Immunosuppressive treatment leading to favorable clinical outcome might be associated with downregulation of immune related pathways. To concretize the transcriptional differences between active disease and remission, a direct comparison was also performed. Repression of genes involved in IL-10 and IL-23 signaling events efficiently discriminated active disease from remission. IL-10 is considered a potent anti-inflammatory cytokine, whereas deregulation of IL-10 signaling pathway might further disturb the deliquate balance between pro- and anti-inflammatory responses in AAV [165].

Specific gene expression perturbancies, which characterize GPA whole blood transcriptome were revealed. Immune-related abberancies, including type 1 IFN and IFNγ signaling pathways, myeloid differentiation, PPR signal transduction and neutrophil degranulation suggest their critical involvement in disease pathogenesis. Unexpectedly, downregulation of genes associated with neutrophil activation was associated with GPA. ANCA activated neutrophil extravasation, immunosuppressive effect of treatment on granulocyte or NETosis might explain this observation [166, 167]. Moreover, activity status of GPA patients was not taken into account; the pathophysiological role of neutrophils might not be precisely depicted. The gene expression of non-neutrophilic cell types in whole blood analysis may interfere, thus making the detection of a specific neutrophilic signature challenging. In agreement with previous studies, upregulation of genes related to myelopoiesis differentiate GPA patients from healthy individuals [144]. Although, granulopoiesis signature in combination with an IFN fingerprint has been previously described in SLE PBMCs [168], a single myeloid differentiation signature without a profound overlapping IFN signature might suggest a highly specific GPA characteristic.

MPA represents a distinct subtype of AAV with increased morbitity and mortality. Transcriptomic analysis of MPA cases demonstrated an upregulation of pathways associated with innate immunity components, such as neutrophil degranulation and MPO. In vitro and in vivo data published suggest that stimuli such as TNF, C5a and bacterial

lipopolysaccharides prime neutrophils, which in turn release ANCA target antigens at cell surface [97-101]. The ANCA-mediated full activation of primed neutrophils follows, which produce destructive enzymes, ROS and extrude NETs [93-109]. A neutophil degranulation signature was also observed which further strengthens previous hypothesis on the pathogenetic role of neutrophils in MPA. Pathways related to cell cycle regulation, DNA damage checkpoints and p53 signaling were also found enriched. Specifically, upregulation of the DNA damage sensor ATM gene and essential for G1/S and G2/M phase transitions *CDK1*, *CDK2* genes was detected. Upon DNA damage (most commonly double strand breaks), ATM binds at the site of damage and induces phoshorylation of CHK2 protein, which in turn stabilizes p53, leading to G1 phase cell cycle arrest [169, 170]. When cell has passed from G1 to S phase, CHK2 inhibits CDC25C phosphatase preventing cell from entering in M phase [169, 170]. Deconvolution analysis is necessary to unmask the contribution of different cell types in cell cycle related transcriptional deregulation and make reasonable assumptions regarding MPA pathogenesis.

Athough GPA and MPA represent two distinct disease subsets; they share common clinical and histological features. This study revealed whole blood transcriptomic differences between GPA and MPA. As one would expect, the upregulation of *MPO* efficiently discriminates MPA from GPA. Moreover, differential expression of genes related to neutrophil degranulation, type 1 and IFNγ signaling was observed. These data might imply the remarkable heterogeneity in the immune mechanisms underlying GPA and MPA. Notably, enrichment of pathways related to cell cycle checkpoints regulation, p53 signaling and biological proceeses relevant to platelet function suggested novel mechanisms, which may broadly differ between the two clinical entities. However, lack of adjustment of factors, such as disease activity or commorbitities may influence the observed results. Additionally, previously mentioned whole blood transcriptomic analysis cannot sufficiently distinguish transcriptional alterations derived from specific cell types, necessitating a further cell type specific sequencing or a deconvolution analysis to make accurate mechanistic hypotheses.

EGPA is a granulomatous vasculitis, which often occurs in patients with previous history of allergic manifestations. According to the data, EGPA transcriptome is characterized by essential pertubarbation in type 1 IFN and cytokine signaling pathways. Interestingly, transcriptional disturbance of neutrophils' functions was not detected. An enrichment analysis of VEGF and intracellular signaling was observed; suggesting that deregulation of angiogenesis may contribute to clinical phenotype. An unexpected downregulation of *IL-5RA* was observed, which is highly expressed by eosinophils and plays a crucial role in the pathogenesis of eosinophilic asthma [171, 172]. The effect of treatment on eosinophils and the analysis of both active and remission EGPA patients might explain the absence of an atopic signature. Noteworthy, an enrichment of pathways linked with CNS cell migration and cellular motility was observed, but further studies are required to support these findings.

We investigated whether whole blood transcriptomic profile can differentiate AAV with active renal disease from active LN. By perforiming PCA, AAV patients were clearly separeated from SLE subjects. This finding might indicate that extensively different pathophysiological mechanisms dictate the two clinical entities. However, technical issues,

such as distinct RNA sequencing methods or variability regarding confounding factors may interfere with our results.

This study has also certain limitations. Firstly, there is a lack of matched patients for disease activity and commorbitities which may interfere with the results in inter-disease analyses. The vast majority of patients involved in this study were receiving immunosuppressive treatment at sampling, including high dose of corticosteroids. Therapy induced immune suppression may be mirrored in AAV whole blood transcriptional profile, preventing the detection of essential pathophysiological mechanisms. Limitation associated with whole blood transcriptomic analysis should also be taken into consideration. Specifically, cellular heterogeneity might mask cell type specific gene signature of pathogenetic importance. Furthermore, longitudinal study is necessary to precisely determine intra-individual transcriptional changes. As this study included predominantly Caucasians, generalization of our results to other ethnic groups is questionable.

More-strict eligibility criteria regarding patient enrollment could be applied to silence the effect of potential confounding factors in this study. By analysing the transcriptional profile of newly diagnosed AAV patients, who did not receive any immunosuppressive treatment novel pathphysiological processes might be elucidated. Additionaly, the transcriptional fingerprint of specific treatment choices (rituximab, cyclophosphamide) could be analysed in a new cohort of AAV patients. Moreover, although our sample size was adequate to identify transcriptomic aberrations of specific disease subsets, a larger number of AAV patients and healthy individuals is required to enhance the accuracy of these findings. qPCR validation of a subset of identified genes, which are involved in type 1 IFN, IFNγ, neutrophil degranulation and cell cycle checkpoints pathways is also required to confirm detected transcriptional disturbancies. Deconvolution analysis is considered an easily applicable tool to detect whether a gene expression variation reflects a shift in cell populations, a change of cell-type-specific expression, or both. We could use this tool to estimate proportion of immune cell subsets in this data. Sequencing of major whole blood cell types might also dramatically augment the specificity of our results. Cell type specific sequencing may allow identification of minor transcriptional differences, which might shed light on novel pathogenetic mechanisms or suggest new targets for therapeutic interventions.

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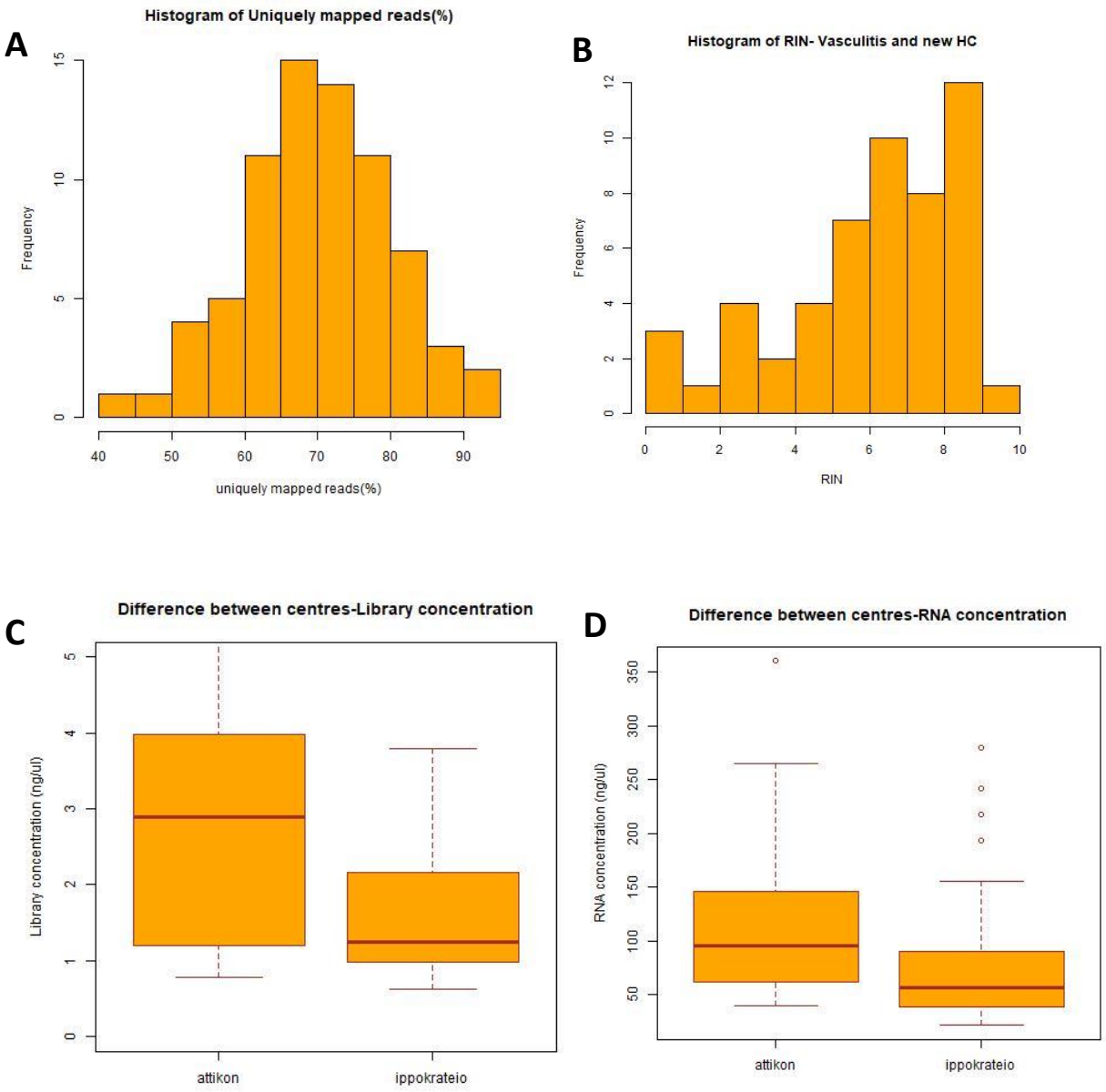
Supplementary



Supplementary 1. Sequencing Characteristics. (A) Histogram of the percentage of the uniquelly mapped reads of samples (B) Histogram of the RIN of samples (C) Boxplots of library concentration - Attikon and Hippokration (p=0.001956) (D) Boxplots of RNA concentration - Attikon and Hippokration (p=0.007214)