School of Health Sciences
SCHOOL OF MEDICINE
Department of Clinical and Laboratory Medicine
Department of Pathophysiology
Director: Professor Michael Voulgarelis

HELLENIC REPUBLIC
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Extensive phenotyping of vascular damage in non-infectious vasculitides with the use of non-invasive vascular biomarkers: prevalence, pathogenesis and response to treatment

DOCTORAL DISSERTATION

Ourania D. Argyropoulou
Medical Doctor, Rheumatologist

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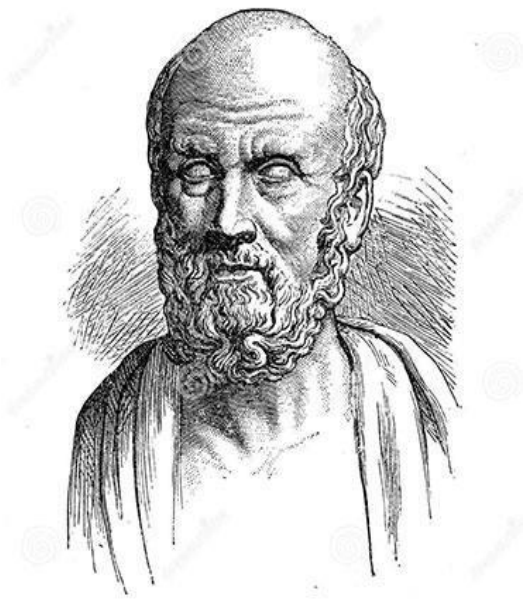

Ὄμνυμι Ἀπόλλωνα ἰητρὸν, καὶ Ἀσκληπιὸν, καὶ Ὑγείαν, καὶ Πανάκειαν, καὶ θεοὺς πάντας τε καὶ πάσας, ἵστορας ποιεύμενος, ἐπιτελέα ποιήσειν κατὰ δύναμιν καὶ κρίσιν ἐμὴν ὅρκον τόνδε καὶ ξυγγραφὴν τήνδε. Ἡγήσασθαι μὲν τὸν διδάξαντά με τὴν τέχνην ταύτην ἴσα γενέτῃσιν ἐμοῖσι, καὶ βίου κοινώσασθαι, καὶ χρεῶν χρηίζοντι μετάδοσιν ποιήσασθαι, καὶ γένος τὸ ἐξ ωὐτέου ἀδελφοῖς ἴσον ἐπικρινέειν ἄῤῥεσι, καὶ διδάξειν τὴν τέχνην ταύτην, ἢν χρηίζωσι μανθάνειν, ἄνευ μισθοῦ καὶ ξυγγραφῆς, παραγγελίης τε καὶ ἀκροήσιος καὶ τῆς λοιπῆς ἁπάσης μαθήσιος μετάδοσιν ποιήσασθαι υἱοῖσί τε ἐμοῖσι, καὶ τοῖσι τοῦ ἐμὲ διδάξαντος, καὶ μαθηταῖσι συγγεγραμμένοισί τε καὶ ὡρκισμένοις νόμῳ ἰητρικῷ, ἄλλῳ δὲ οὐδενί. Διαιτήμασί τε χρήσομαι ἐπ' ὠφελείῃ καμνόντων κατὰ δύναμιν καὶ κρίσιν ἐμὴν, ἐπὶ δηλήσει δὲ καὶ ἀδικίῃ εἴρξειν. Οὐ δώσω δὲ οὐδὲ φάρμακον οὐδενὶ αἰτηθεὶς θανάσιμον, οὐδὲ ὑφηγήσομαι ξυμβουλίην τοιήνδε. Ὁμοίως δὲ οὐδὲ γυναικὶ πεσσὸν φθόριον δώσω. Ἁγνῶς δὲ καὶ ὁσίως διατηρήσω βίον τὸν ἐμὸν καὶ τέχνην τὴν ἐμήν. Οὐ τεμέω δὲ οὐδὲ μὴν λιθιῶντας, ἐκχωρήσω δὲ ἐργάτῃσιν ἀνδράσι πρήξιος τῆσδε. Ἐς οἰκίας δὲ ὁκόσας ἂν ἐσίω, ἐσελεύσομαι ἐπ' ὠφελείῃ καμνόντων, ἐκτὸς ἐὼν πάσης ἀδικίης ἑκουσίης καὶ φθορίης, τῆς τε ἄλλης καὶ ἀφροδισίων ἔργων ἐπί τε γυναικείων σωμάτων καὶ ἀνδρῴων, ἐλευθέρων τε καὶ δούλων. Ἃ δ' ἂν ἐν θεραπείῃ ἢ ἴδω, ἢ ἀκούσω, ἢ καὶ ἄνευ θεραπηίης κατὰ βίον ἀνθρώπων, ἃ μὴ χρή ποτε ἐκλαλέεσθαι ἔξω, σιγήσομαι, ἄῤῥητα ἡγεύμενος εἶναι τὰ τοιαῦτα. Ὅρκον μὲν οὖν μοι τόνδε ἐπιτελέα ποιέοντι, καὶ μὴ ξυγχέοντι, εἴη ἐπαύρασθαι καὶ βίου καὶ τέχνης δοξαζομένῳ παρὰ πᾶσιν ἀνθρώποις ἐς τὸν αἰεὶ χρόνον. παραβαίνοντι δὲ καὶ ἐπιορκοῦντι, τἀναντία τουτέων.

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Curriculum Vitae

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Characterization of Senescent Cells in Temporal Arteries of Patients with Giant Cell Arteritis Reveal an Inflammatory Phenotype and Strong Dependence from IL-6.
Veroutis D, **Argyropoulou O**, Goules A, Kambas K, Palamidas DA, Evangelou K, Havaki S, Polyzou A, Xingi E, Karatza E, Boki K, Cavazza A , Kittas C, Thanos D, Ricordi C, Marvisi C, Muratore F, Galli E, Croci S, Salvarani C, Gorgoulis VG, Tzioufas AG.
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1H-NMR BASED METABOLOMIC PROFILE OF PATIENTS WITH GIANT CELL ARTERITIS AND POLYMYALGIA RHEUMATICA IN ACTIVE AND INACTIVE DISEASE STATE
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8th Two-day conference on the topic: Vaculitis, Athens, Greece 2022.

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The differences in the clinical spectrum of Cryoglobulinemic Vasculitisbetween Sjögren's syndrome and HCV hepatitis.
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27th Panhellenic Congress on Rheumatology (Virtual) 2020.

POSTERS IN CONGRESSES

Characterization of Senescent Cells in Temporal Arteries of Patients with Giant Cell Arteritis Reveal an Inflammatory Phenotype and Strong Dependence from IL-6.
Veroutis D, Argyropoulou O, **Goules A**, Kambas K, Palamidas DA, Evangelou K, Havaki S, Polyzou A, Xingi E, Karatza E, Boki K, Cavazza A , Kittas C, Thanos D, Ricordi C, Marvisi C, Muratore F, Galli E, Croci S, Salvarani C, Gorgoulis VG, Tzioufas AG.
American College of Rheumatology (ACR) Convergence 2023, San Diego, U.S.A.

1H-NMR BASED METABOLOMIC PROFILE OF PATIENTS WITH GIANT CELL ARTERITIS AND POLYMYALGIA RHEUMATICA IN ACTIVE AND INACTIVE DISEASE STATE.
Argyropoulou OD, Karagiannakou M, Palamidas DA, Benaki D, Tsezou K, Vlachoyiannopoulos PG, Mikros E, Tzioufas AG.
EUROPEAN CONGRESS OF RHEUMATOLOGY (EULAR) 2022, Copenhagen, Denmark.

Kinetics of Mononuclear Cell Subpopulations in the Peripheral Blood of Patients with Giant Cell Arteritis During the Acute Phase of the Disease: The Role of Steroids.
Palamidas DA, Argyropoulou OD, Paschalidis N, Sideras P, Tzioufas AG
28th Panhellenic Congress on Rheumatology, Athens, Greece.

IDIOPATHIC OPHTHALMIC MYOSITIS: CLINICAL PRESENTATION, THERAPEUTIC APPROACH AND IMAGING DIFFERENTIATION FROM GRAVES OPHTHALMOPATHY

Ziaragkali S, Argyropoulou O, Boutzios G, Chatzi S, Papadaki E, Karantanas A, Tzioufas A, Manoussakis MN.
28th Panhellenic Congress on Rheumatology, Athens, Greece.

Specificity of pANCA autoantibodies in autoimmune diseases.
Argyropoulou OD, Tsirogianni A, Sfontouris C, Boutzios G, Vlachoyiannopoulos PG, Tzioufas AG, Kapsogeorgou EK.
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Argyropoulou OD, Aissopou EK, Argyris A, Goules AV, Mavragani CP, Tentolouris N, Sfikakis PP, Vlachoyiannopoulos P, Tzioufas AG, Protogerou A.
EUROPEAN CONGRESS OF RHEUMATOLOGY (EULAR) 2021, Virtual Congress.

The differences in the clinical spectrum of Cryoglobulinemic Vasculitis between Sjögren's syndrome and HCV hepatitis.
Argyropoulou O, Pezoulas V, Quartuccio L, Ferro F, Gandolfo S, Donati V, Venetsanopoulou A, Chatzis L, Zampeli E, Mavromati M, Voulgari P, Mavragani CP, Baldini C, Skopouli F, Fotiadis D, Galli M, De Vita S, Moutsopoulos HM, Goules A, Tzioufas A.
EUROPEAN CONGRESS OF RHEUMATOLOGY (EULAR) 2020, Virtual Congress.

The clinical features of Sjögren's syndrome patients with early and late disease onset.
Goules A, Argyropoulou O, Pezoulas V, Ferro F, Gandolfo S, Donati V, Binutti M, Callegher SZ, Chatzis L, Venetsanopoulou, Zampeli E, Mavromati M, Voulgari P, Mavragani C, Baldini C, Skopouli F, Fotiadis D, De Vita S, Moutsopoulos HM, Tzioufas A.
EUROPEAN CONGRESS OF RHEUMATOLOGY (EULAR) 2020, Virtual Congress.

TEN-YEAR OVERALL SURVIVAL AND STANDARIZED MORTALITY RATIO IN THE LARGEST SINGLE CENTER COHORT OF PATIENTS WITH PRIMARY SJOGREN'S ASSOCIATED LYMPHOMAS.
Sikara M, Ziogas D, **Argyropoulou O**, Papageorgiou A, Tzioufas A, Voulgarelis M.
EUROPEAN CONGRESS OF RHEUMATOLOGY (EULAR) 2019, Madrid, Spain.

ANALYSIS OF CLINICAL AND SEROLOGICAL PICTURE OF PATIENTS WITH PRIMARY SJöGREN'S SYNDROMEAND AN EARLY DISEASE ONSET AT AGE BEFORE 35 YEARS.
Argyropoulou O, Goules A, Zampeli E, Mavromati M, Mavragani C, Skopouli F, Moutsopoulos HM, Tzioufas A.
EUROPEAN CONGRESS OF RHEUMATOLOGY (EULAR) 2019, Madrid, Spain.

THE CLINICAL SPECTRUM AND PEDIGREE ANALYSIS OF TRAPS IN GREECE, INCLUDING A NOVEL MUTATION-RESULTS FORM A NATIONAL REFERRAL CENTRE.

Nezos A, **Argyropoulou O**, Klinaki E, Marketos N, Panagiota K, Maritsi D, Vlachoyiannopoulos P, Tzioufas A.
EUROPEAN CONGRESS OF RHEUMATOLOGY (EULAR) 2019, Madrid, Spain.

CLINICAL FEATURES, TREATMENT MODALITIES AND RELAPSE RATES IN GREEK PATIENTS WITH RETROPERITONEAL FIBROSIS FREE
Venetsanopoulou A, Zampeli E, Christaki S, Argyropoulou O, Boki KA, Manoussakis MN, Skopouli FN, Tzioufas A, Moutsopoulos HM.
EUROPEAN CONGRESS OF RHEUMATOLOGY (EULAR) 2019, Madrid, Spain.

CLINICAL TRIALS

2023- ongoing (Sub investigator): ARGX-113-2007: A phase2/3, study to investigate the efficacy and safety of efgartigimod PH20 SC in adults with active idiopathic inflammatory myopathy.

2021-2023 (Sub investigator): TWIISS and TWINSS extension trial: TWIISS and TWINSS extension trial to evaluate the safety and tolerability of CFZ533 (Iscalimab) at two dose levels administered subcutaneously in patients with Sjögren's syndrome.

2021-2022 (Sub investigator): GLPG3970 trial: A randomized, double-blind, placebo-controlled, multicenter study to evaluate the efficacy, safety, tolerability, pharmacokinetics, and pharmacodynamics of orally administered GLPG3970 for 12 weeks in adult subjects with active primary Sjögren's syndrome.

2021 (Sub investigator): PONOS_A4091091: A non-interventional prospective study to evaluate the impact of moderate to severe symptomatic knee/hip osteoarthritis (OA) on the life of affected patients, as well as to the healthcare system.

CERTIFICATES

2023: ICH GOOD CLINICAL PRACTICE E6 (R2) (SCORE 100%)
2019: GCP Compliance Training for HCPs V2.0

INVITED SPEAKER IN CONGRESSES

Imaging of large vessel vasculitis: coupling clinical and translation research.
Argyropoulou O, Inflammation and Thrombosis, 2023, Alexandroupoli, Greece.
IMPORTANT PUBLICATIONS OF THE LAST YEAR-BASIC RESEARCH (YEAR IN REVIEW).
Argyropoulou O, SPRING DAYS OF RHEUMATOLOGY, 2023, Patra, Greece.
DYNAMICS OF METABOLOMIC EXPRESSION AND FUNCTIONAL IMAGING IN SYSTEMIC VASCULITIS
Argyropoulou O, 25th SCIENTIFIC MEETING OF RHEUMATOLOGISTS OF NORTHWEST GREECE, 2023, Ioannina, Greece.

Pathogenesis and Treatment of Systemic Vasculitis: Giant Cell Arteritis.
Argyropoulou O, ANNUAL IMMUNOPATHOLOGY SEMINAR FOR RHEUMATOLOGISTS, 2022, Kalavryta, Greece.

PATHOGENESIS OF GIANT CELL ARTERITIS: WHAT´S NEW
Argyropoulou O, 8th Two-day conference on the topic: Vasculitis, Athens, Greece 2022.

T cells "T be or not T be that is the question"
Argyropoulou O, CIVIS 2022, Athens, Greece

SYTEMIC RHEUMATIC DISEASES (ACR REVIEW 2021)
Argyropoulou O, Post-education courses in Rheumatology 24th series, 2021, Thessaloniki, Greece.

New Pathogenetic Mechanisms and rising Biomarkers in Systemic Vasculitides: Giant Cell Arteritis.
Argyropoulou O, 13th Panhellenic congress of the Scientific Society of Musculoskeletal Health 2021, Xalkidiki, Greece 2021.

PATHOGENETIC ROLE OF NEUTROPHILS IN SYSTEMIC VASCULITIS.
Argyropoulou O, 7th Two-day conference on the topic: Vaculitis, 2021, Athens, Greece.

SYSTEMIC VASCULITIS: FROM PATHOGENESIS TO PERSONALIZED MEDICINE
Argyropoulou O, Messinian day of Rheumatology, 2020, Kalamata, Greece.

Checkpoint Inhibitors and Autoimmunity
Argyropoulou O, 21th SCIENTIFIC MEETING OF RHEUMATOLOGISTS OF NORTHWEST GREECE, 2019, Ioannina, Greece.

NEW PATHOHENETIC DATA: SJOGREN' SYNDROME, SYSTEMIC VASCULITIS, INFLAMMATORY MYOPATHIES.
Argyropoulou O, SPRING DAYS OF RHEUMATOLOGY, 2019, Kalamata, Greece.

AWARDS

1st award by the Scientific Society of Musculoskeletal Health for the best research work for the oral presentation "Characterization of Senescent Cells in Temporal Arteries of Patients with Giant Cell Arteritis Reveal an Inflammatory Phenotype and Strong Dependence from IL-6".
Veroutis D, **Argyropoulou O**, Goules A, Kambas K, Palamidas DA, Evangelou K, Havaki S, Polyzou A, Xingi E, Karatza E, Boki K, Cavazza A , Kittas C, Thanos D, Ricordi C, Marvisi C, Muratore F, Galli E, Croci S, Salvarani C, Gorgoulis VG, Tzioufas AG.

15th Panhellenic congress of the Scientific Society of Musculoskeletal Health Crete, Greece 2023.

By the Greek Rheumatology Society in memory of Alexandre Andrianakos for the oral presentation: "The differences in the clinical spectrum of Cryoglobulinemic Vasculitisbetween Sjögren's syndrome and HCV hepatitis".
Argyropoulou O, Pezoulas V, Quartuccio L, Ferro F, Gandolfo S, Donati V, Venetsanopoulou A, Chatzis L, Zampeli E, Mavromati M, Voulgari P, Mavragani CP, Baldini C, Skopouli F, Fotiadis D, Galli M, De Vita S, Moutsopoulos HM, Goules A, Tzioufas A.
27th Panhellenic Congress on Rheumatology (Virtual) 2020.

DIDACTIC WORK

2017-today: Review of the literature lectures and research meetings. Department of Pathophysiology, NKUA, Athens, Greece.

2021-today: Clinical training and lectures of medical students (at the 6th year of training). Department of Pathophysiology, NKUA, Athens, Greece.

2022 CIVIS, Athens, Greece
Title: T cells "T be or not T be that is the question".

2020-2021: "Internal medicine" of the postgraduate program "Internal medicine, Surgery and Oral dental radiology, Dental Department of NKUA, Athens, Greece
Title: Differential diagnosis of arthritis: from pathogenesis to clinical practice.

LANGUAGES

Greek (Excellent), English (Excellent), German (Very good), French (Very good)

PARTICIPATION IN RESEARCH WORKING GROUPS AND ASSOCIATIONS

2022-today:	Member of the European Reference Network for Rare Immunodeficiency, Autoinflammatory and Autoimmune Diseases (RITA-ERN).
2015-today:	Member of the Greek Rheumatology Society, Greece.
2007-2009 & 2010-today:	Member of the Medical Association of Athens
2009-2010:	Member of the Medical Association of Messinia, Greece.

Abbreviations

AAV:	ANCA-associated vasculitis
AH:	arterial hypertension
AIH:	autoimmune hepatitis
AIP:	atherogenic index of plasma
AIx:	augmentation index
ANA:	antinuclear antibodies
A-ANCA:	atypical ANCA
ANCA:	antineutrophil cytoplasmic autoantibodies
AP:	augmentation pressure
APS:	antiphospholipid syndrome
AZA:	azathioprine
BD:	Behcet's Disease
BMI:	body mass index
BP:	blood pressure
BPI:	bactericidal permeability increasing protein
BVAS:	Bermingham Vasculitis Activity Score
c-ANCA:	cytoplasmic ANCA
cfPWV:	carotid-femoral pulse wave velocity
cIMT:	carotid intima media thickness
CNS:	central nervous system
CRAE:	central retinal arteriolar equivalent
CRP:	c-reactive protein
CRVE:	central retinal venular equivalent
CyC:	cyclophosphamide
CV:	cryoglobulinemic vasculitis
CVD:	cardiovascular disease
DBP:	diastolic blood pressure
DCs:	dendritic cells
DM:	diabetes mellitus
DTH:	Delayed Type Hypersensitivity

EGCS:	ectopic germinal center like structures
EGPA:	eosinophilic granulomatosis with polyangiitis
ESR:	erythrocyte sedimentation rate
18 FDG PET/CT:	18-fluorodeoxyglucose
GCA:	giant cell arteritis
GCs:	glucocorticosteroids
GPA:	granulomatosis with polyangiitis
GWAS:	genome-wide association studies
HBV:	hepatitis B virus
HCQ:	hydroxychloroquine
HCV:	hepatitis C virus
HDL:	high density lipoprotein
HIV:	human immunodeficiency virus
HLA:	human leukocyte antigen
HR:	heart rate
HSP:	Henoch Schönlein purpura
HT:	Hashimoto thyroiditis
IBD:	inflammatory bowel disease
IFN:	interferon
IIF:	indirect immunofluorescence
IL:	interleukin
ILD:	interstitial lung disease
IMT:	intima media thickness
KD:	Kawasaki disease
LDL:	low density lipoprotein
LR:	logistic regression
LVV:	large vessel vasculitis
MAP:	mean arterial pressure
MI:	myocardial infarction
MICA:	MHC class I polypeptide-related sequence A
MMPs:	matrix metalloproteinases
MP:	membranoproliferative

MPA:	microscopic polyangiitis
MPO:	myeloperoxidase
MTX:	methotrexate
MVV:	medium vessel vasculitides
NETs:	neutrophil extracellular traps
NHL:	non-Hodgkin's lymphoma
NIC:	non-inflammatory controls
NIH:	national institute of health
NK:	natural killer cells
NKUA:	National and Kapodistrian University of Athens
NMR:	nuclear magnetic resonance
PAN:	polyarteritis nodosa
p-ANCA:	perinuclear-ANCA
PBC:	primary biliary cirrhosis
PDGF:	platelet derived growth factor
PET/CT:	positron-emission tomography with computed tomography
RA:	rheumatoid arthritis
RF:	rheumatoid factor
PMR:	polymyalgia rheumatica
PNS:	peripheral nervous system
PR3:	proteinase 3
pSS:	primary Sjögren's syndrome
PSV:	primary systemic vasculitides
PWA:	pulse wave analysis
PWR:	pulse wave reflexions
PWV:	pulse wave velocity
RA:	rheumatoid arthritis
RTX:	rituximab
SARDs:	systemic autoimmune rheumatic diseases
SBP:	systolic blood pressure
SD:	standard deviation
SGE:	salivary gland enlargement

SLE:	systemic lupus erythematosus
SSA:	Sjögren's-syndrome related antigen type A
SSB:	Sjögren's-syndrome related antigen type B
SSCL:	systemic sclerosis
SV:	systemic vasculitides
SVV:	small vessel vasculitis
TAB:	temporal artery biopsy
TAK:	Takayasu arteritis
TCHOL:	total cholesterol
TG:	triglycerides
Th:	T helper lymphocytes
TIMP:	tissue inhibitor of metalloproteinase
TGFβ:	transforming growth factor-beta
TLRs:	toll like receptors
TNF:	tumor necrosis factor
TNFα:	tumor necrosis factor alpha
Tregs:	T regulatory cells
USG:	ultrasonography
VDI:	vasculitis damage index
VVV:	vasa vasorum vasculitis

PART I. GENERAL BACKGROUND

CHAPTER 1. SYSTEMIC VASCULITIDES

1.1. Introduction

Blood vessels constitute the peripheral part of the circulatory system that flow blood throughout the human body, providing nourishment and help in fighting diseases and maintaining homeostasis. Vascular destruction compromises the sustainability of life since all human organs rely on their functionality. The inflammatory endothelial injury is a major cause of cardiovascular morbidity and mortality in the general population, but still not adequately understood.

Systemic Vasculitides (SV) represent a heterogeneous group of systemic, chronic and potentially life-threatening autoimmune/autoinflammatory diseases of unknown etiology, which serve as an excellent disease model for CVD of inflammatory origin. Their hallmark is the partial, focal or diffuse inflammatory mediated affection of any type (arteries, veins) and size (aorta and its main branches to arterioles, venules and capillaries) of blood vessels, leading, if untreated, to their destruction. The inflamed vascular wall becomes prone to be occluded, form aneurysms, rupture or develop a thrombus, thus leading to ischemia and necrosis of multiple organs including kidneys, lungs, central nervous system, heart, eyes, musculoskeletal and skin. They can be primary or develop in the setting of other medical conditions including infections (e.g. hepatitis C, HIV), systemic autoimmune rheumatic diseases (e.g. rheumatoid arthritis, systemic lupus erythematosus, seronegative spondyloarthropathies, Sjögren's syndrome), malignancies (e.g. lymphomas, myelodysplastic syndromes, solid tumors) and drugs (e.g. propylthiouracil, hydralazine, leukotriene receptor antagonists). The Chapel Hill Consensus Conference proposed a nomenclature system which subdivides vasculitides according to vessel size (large, medium and small) predominantly involved and the association with ANCAs (1).

Although their prevalence varies significantly among different ethnic groups, they are uncommon but not rare in Western countries affecting overall 1 in 2000 adults. All age groups can be affected extending from childhood and early adulthood (e.g KD, HSP, TAK) to middle aged and elderly individuals (AAV, GCA and PAN) (2). According to

previous studies systemic vasculitides have a 12% mortality, whereas their recurrence rate ranges from 10 to 50% (Table 1.1) (3). In necrotic SVV, premature deaths are mainly attributed to microvascular complications from the kidneys (acute renal insufficiency) and the lungs (pulmonary hemorrhage) (4) whereas, in medium and large vessel predominate the macro-vascular (e.g. blindness, coronary heart disease, stroke, formation and / or rupture of aneurysms). Patients with GCA have 6.6-17.3 increased risk of developing thoracic aortic aneurysms with the incidence increasing 5 years after the disease diagnosis and continue to increase thereafter (5).

SV	*Annual Incidence rates per million*	*Ethnicity*	*Mean Age of Onset (years)*	*Female / Male*	*5-year survival rate (%)*
GCA	100-216	White North Europeans	≥ 50	2-3:1	67-85
TAK	0.4-3.4	Southeast Asia	20-40	8:1	80-90
PAN	0.9-8.8	All racial groups	40-60	1:1.5	65-75
GPA	2.1-14.4	Northern Europe	40-50	1:1	74-91
MPA	2.7-10.4	Southern Europe	50-60	1:1	45-76
EGPA	0.14-4.0	All racial groups	40-60	1:2	60-97

Table 1.1 *Epidemiologic features and outcome of the major Systemic Vasculitides (2,3,4,5).*

1.2. Pathogenesis

Although the etiology and pathogenesis of Systemic Vasculitides are far from being completely understood, cumulating data suggest a persistent interplay of innate and adaptive immune responses incited most probably by an environmental trigger (e.g. silica, Hepatitis B virus in PAN, Staphylococcus aureus in GPA) in genetically predisposed individuals. Large-scale genetic analyses using the Immunochip platform and GWAS facilitated the identification of shared, as well as unique etiopathogenic pathways among

SV	Susceptibility locus	SNP
GCA	HLA-DRB1, HLADQA1	rs9405038
	PLG	rs4252134
	P4HA2	rs128738
	KDM4C	rs16925200
	IL12B	rs755374
	NOS2	rs7207044
	CEP128	rs61981699
	ZMIZ1	rs1250544
	KIAA1841	rs115069423
	RGS21/RGS1	rs12725829
TAK	HLA-B/MICA	rs12524487
	KDM4C	rs16925200
	IL12B	rs755374
	NOS2	rs7207044
	CEP128	rs61981699
	ZMIZ1	rs1250544
	KIAA11841	rs115069423
	RGS21/RGS1	rs12725829
	FCGR2A	
	LILR3B	
PAN	CECR1	
GPA	*HLA DPB1*0401, HLA B50, HLA DR9, HLA DQw7, HLA DR3, HLADR1, HLADR4, HLADR6*	rs6679677
	PTPN22	rs41295061
	IL-2RA	rs3087243
	CTLA4	rs62132295
	PRTN3	
	AAT Z Allele	rs1790947
	CD226	rs16925200
	KDM4C	rs7151526
	SERPINA1	rs3759467
	TNFSF13B	
	FCGR3B	rs62132295
	PRTN3	rs3130233
	COL11A2	rs3117016
	COL11A2	rs1554286
	L10	rs2070947
	ITGB2	rs1250544
	ZMIZ1	rs115069423
	KIAA1841	rs12725829
	RGS21/RGS1	
MPA	*HLA DQw7, HLA DR3,HLADR4, HLADR6*	
	PTPN22	rs6679677
	IL-2RA	rs41295061
	CTLA4	rs3087243
	CD226	rs1790947
	KDM4C	rs16925200
	SERPINA1	rs7151526
	TNFSF13B	rs3759467
	LILRA2	
	FCGR3B	
	PRTN3	rs62132295
	COL11A2	rs3130233
	COL11A2	rs3117016
	L10	rs1554286
	ITGB2	rs2070947
	ZMIZ1	rs1250544
	KIAA1841	rs115069423
	RGS21/RGS1	rs12725829
EGPA	*HLA DRB4, HLA DRB3*	
	HLA-DQ	rs9274704
	PTPN22	rs6679677
	IL-2RA	rs412295061
	CTLA4	rs3087243
	KDM4C	rs16925200
	TNFSF13B	rs3759467
	BCL2L11	rs72946301
	TSLP	rs1837253

LVV (6) and AAV, supporting the hypothesis of a partially common genetic background beyond auto-immunity (Table 1.2). Shared loci that have been described include RGS21/RGS1, PRKCQ, TNFSF15, NDFIP1, KIAA1841, ZMIZ1 and SERBP1. Furthermore, different SNPs have also been associated with disease expression with the most strongly associated being the KDM4C SNP rs16925200 (7). Interestingly, the polymorphism located at 5' end of IL12B rs755374 represents the most common associated SNP shared between GCA and TAK. This gene encodes the p40 subunit of IL12 and IL23, participating in Th1 and Th17 differentiation. These cells are considered pivotal players in the development of LVV. On the contrary, high heterogeneity is observed across class I and class II regions.

Table 1.2 *Indicative Genetic loci associated with SV.*

Indeed, GCA is mostly associated with class II genes, with the SNP rs 9405038 (located between HLA-DRA and HLA-DRB1) representing the lead signal whereas the main associations with TAK are located within the class I sub region with rs 12524487 (located between HLA-B and MICA). Similarly, distinct genetic associations between disease subtypes have been identified among AAV (8). HLA, SERPINA1 and PRTN3 are primarily aligned with ANCA specificity rather than with the clinically defined GPA and microscopic polyangiitis MPA (9).

Beyond the genetic background the pathogenic operating mechanisms involved in inflammatory vascular damage are: a) activation and dysfunction of the endothelium, b) production of autoantibodies against surface antigens of the endothelial cells or neutrophil fragments with the typical example of ANCA associated vasculitides (10), c) the chronic delayed T-mediated immune damage (DTH) (11), d) the detrimental effect of NETs (12), e) immune complex (IC) mediated disease (cryoglobulinemic vasculitis is considered the prototype IC disease) (114), and f) direct action of antibodies against tissue specific autoantigens (Goodpasture) (13). Large vessel vasculitides are predominantly cell mediated. The vasa vasorum in the adventitia is probably the initial site of insult. Activation of DCs via TLRs by an unknown trigger induces the production of IL-18, IL-12, IL-23 and chemokines (e.g. CCL19, CCL21) enhancing in turn the influx of inflammatory cells, making eventually sustainable the aberrant autoimmune response. TLR4 ligands trigger the recruitment of CD4+ T-cells that infiltrate all layers of the arterial wall producing a transmural panarteritis, whereas TLR5 ligands are associated with a more limited form of the disease, apparent as adventitial perivascular infiltrate (14). Studies on T-cells revealed that, CD4+ T-cells proliferate to form Th1, Th17 and Tregs. The production of IFNγ and IL-2 by Th1 as well as IL-17 by Th17 enables activated macrophages which produce IL-1, IL-6, TNFα and TGF-β to infiltrate the media, form giant cells and secret MMPs leading to the destruction of the internal elastic lamina and eventually to neovascularization. This change of the tissue, along with the assistance of certain cytokines including PDGF and IL-1 contribute to vascular remodeling by inducing myofibroblast activation and overproduction of matrix proteins (15). A subset of T-lymphocytes producing IL-9 (TH9 cells) has also been proposed to play a role in the pathogenesis of LVV displaying a

characteristic pattern of transmural inflammation and small vessel vasculitis (16). Contrary to LVV the key cell population in AAV is neutrophils. Unknown environmental triggers account for the release of inflammatory cytokines (e.g. IL-1, TNF-α) as well as upregulation of adhesion molecules on endothelial cells, resulting in the excessive activation of neutrophils and monocytes which bind and transmigrate into the vessel wall. Activated and committed neutrophils produce reactive oxygen species while their degranulation within tissue injury releases lysosomal enzymes lead to endothelial injury. Neutrophils are forming also NETs decorated with IL-1 that may help in the perpetuation of inflammatory responses (12). Degranulation of the circulating neutrophils releases reactive oxygen species and lysosomal enzymes leading to endothelial injury. Dendritic cells, macrophages, B cells and T cells, the complement system and humoral factors are also involved in the pathogenesis of AAV. In addition to specific immune mediated inflammatory pathways towards vascular injury, the acceleration of the classical pathways of arterial damage leading to, atheromatosis (i.e., atheromatic plaque formation), inappropriate arterial remodeling (e.g., arterial hypertrophy / distention) and arteriosclerosis (i.e., arterial stiffening), are also thought to participate in the development of the micro- and macrovascular complications of these diseases (17). Thus, the vessels constitute the venue where both inflammatory, arterial remodeling and atheromatosis pathways meet, interact, and give rise to different disease phenotypes.

1.3. Clinical picture

The size, localization of the affected vessels and the ischemia of the irrigated organs in association with the vast array of the underlying mechanisms participating in the inflammatory process, account for the wide spectrum of clinical manifestations (Figure 1.1). SV are the second cause of fever of unknown origin in middle and older individuals, imposing sometimes long-term hospitalization of patients until the final diagnosis. They should be suspected in any constitutionally ill patient with evidence of multisystem inflammatory disease. The onset might be subacute with nonspecific signs and symptoms unfolding slowly over weeks to months or acute, leading to

rapidly progressive and usually terminal organ damage and failure, making the diagnosis of vasculitis one of the great diagnostic challenges in clinical Medicine. This is further suggested by the existence of various distinct, as well as overlapping clinical subsets within each form of vasculitis. GCA patients can present with at least four different phenotypes including cranial ischemia, systemic inflammation, vasculitis of large arteries (e.g. aorta, subclavian, axillary arteries), polymyalgia rheumatica or combination (18). PAN is sub dived into mild or severe cutaneous with ulcers, necrosis, and neuritis as well as systemic vasculitis resulting in vascular occlusion and aneurysmal formation (19). AAV are also characterized by clinical heterogeneity. GPA can be limited or generalized involving the upper respiratory tract, lungs, and kidneys. Similarly, MPA and EGPA can cause necrotizing vasculitis of single or multiple organs in association with constitutional symptoms (20). Distinguishing between the different clinical phenotypes as well as with other inflammatory conditions of internal medicine is of pivotal value since treatment varies dramatically and misdiagnosis may result in increased morbidity and mortality.

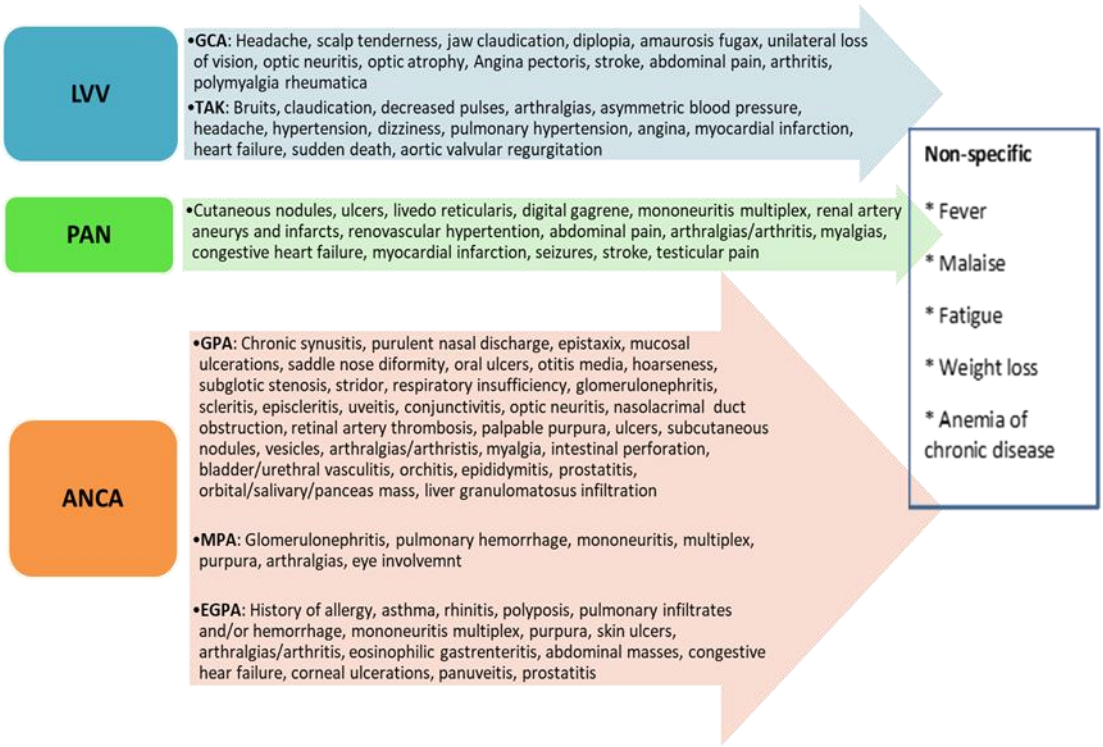

Figure 1.1 *Clinical features of Systemic Vasculitides*

1.4 Diagnosis

The final diagnosis and the clinical phenotype of the disease is a complicated procedure and a constant concern for clinicians since it requires confirmation by serologic tests, a biopsy of an involved artery or tissue and the use of multiple imaging tools. After the clinical suspicion of SV, the diagnostic procedures should be performed quickly, since delayed diagnosis imposes hospitalization in intensive care units, thus increasing the consequent disability, mortality rate and the financial burden on the National Health System. All the so far existing laboratory tests (Table 1.3) are not highly disease specific but only suggestive of SV in the proper clinical setting. Some reflect systemic inflammation, while others suggest organ involvement, immune complex formation, and mechanistic pathways (21).

Table 1.3. *Laboratory tests useful in the evaluation of Vasculitides*

Systemic inflammation	***ANCA Autoantibodies***
Complete blood count	c-ANCA
Anemia of chronic disease	anti-PR3 (66% GPA, 26% MPA,
Neutrophilia	10% EGPA)
Eosinophilia	p-ANCA
Thrombocytosis	anti-MPO (60% MPA, 50% EGPA,
Acute phase reactants	15-30% GPA, 10-20% CTD, drugs)
ESR >50 mm	Atypical
CRP > 10 mg/dl	
Hypoalbuminemia	
Organ involvement	***Suggesting etiology***
Creatinine and urinalysis	Blood cultures
Liver-associated enzymes	Infectious serologies
Creatine kinase	Hep BsAg (PAN)
Stool for occult blood	Hepatitis C (cryoglobulinemia)
	Parvovirus IgM (GPA, PAN)
	Cytomegalovirus (IgM),
Immune complex formation and/or deposition	Epstein Barr virus (IgM)
RF, ANA (should be negative in primary Systemic	HIV1/2 (any vasculitis)
Vasculitides)	Serum protein electrophoresis
Complement levels (C3/C4) (usually normal,	Cerebrospinal fluid studies
low in 25% of PAN)	Urinary toxicology screen (rule out
Cryoglobulins	cocaine use)

ANCA subtypes (proteinase-3 (PR3)-ANCA and myeloperoxidase (MPO)-ANCA) are associated with different epidemiological, genetic, and clinical features. Among GPA patients with positive ANCA, 80-90% are c-ANCA with anti-PR3 specificity and only 10%-20% are p-ANCA directed against MPO. The overall specificity is for generalized

GPA 98% and for the limited form approximately 60%. ANCA titers correlate with disease activity in 60% of cases, whereas 40% of GPA patients with inactive disease may have a rise in ANCA titer. Therefore, a transition of ANCA titers from negative to positive is better predictor of disease flare than a mild rise in an already positive serum (22). p-ANCA directed against MPO is found in 60% of MPA and in 30-70% of EGPA patients especially in those with renal disease, alveolar hemorrhage, mononeuritis multiplex and purpura (23).

Tissue biopsy is the procedure of choice in the diagnosis and classification of SV. Beyond diagnosis, it provides important information, including the extension and phenotype of the disease, the composition of inflammatory cells and the acute or chronic changes of the affected organ. TAB is the gold standard for the diagnosis of GCA, although a negative TAB does not exclude the presence of the disease. The characteristic patchy or segmental arterial involvement is the predominant cause for a negative biopsy in the context of high clinical suspicion. GCA patients with aortitis and other large vessel involvement have a negative TAB in approximately 40% of cases. The primary site of pathology is thought to be the vasa vasorum with subsequent inflammatory spreading from the adventitia into the vessel wall. The histologic heterogeneity of the disease is depicted in four different patterns and may have prognostic value regarding the clinical course and response to treatment (24).

Although, all untreated patients irrespectively to the biopsy pattern can develop blindness in up to 15%, SVV pattern correlates with less severe clinical manifestations, lower serum levels of acute phase reactants and a milder disease course than VVV or the classic patterns. Polymyalgia rheumatica is equally represented in all patients' groups, while male sex and peripheral arthritis are seen more frequently in SVV (24). In TAK the primary site of pathology appears to be in the adventitia and outer parts of the media, where the resident dendritic cell is activated from unknown factors, mounting an aberrant immune response involving all arms of cellular immunity (25). Until recently, the diagnosis of TAK was established mainly on the basis of angiographic features. Besides, being an invasive technique bearing the risk of allergic reactions, iatrogenic embolization and arterial dissection, angiography visualizes luminal changes caused by vasculitis (e.g stenosis, occlusion, aneurysmal formation) but cannot

delineate vessel wall pathology. More currently applied imaging modalities (Figure 1.2) constitute useful diagnostic tools for Systemic Vasculitides. According to European League Against Rheumatism (EULAR) recommendations for the management of vasculitis, imaging should be performed as early as possible in patients with suspected LVV, particularly in GCA, because pathologic findings often become less apparent or even false negative following steroid treatment. This is particularly the case in the imaging of temporal arteries with ultrasound (U/S) or magnetic resonance imaging (MRI) and with positron emission tomography (PET) of extracranial vessels. Ultrasound and MRI are the first choices in cranial GCA and TAK, respectively. Computed tomography with PET (PET/CT) may be used to investigate extracranial disease (26). U/S of temporal and axillary arteries is recommended as the first imaging modality in GCA with 62-87% sensitivity and 85-99% specificity. Low echogenicity associated with increased wall thickness or new stenosis at follow up might indicate active disease. However, in most cases, U/S is only possible to monitor damage. High resolution MRI of cranial arteries may be used as an alternative for GCA if ultrasound is not available or inconclusive. Overall, MRI exhibits 57-87% sensitivity and 81-92% specificity and is superior compared to U/S in assessing aortic inflammation and periaortitis. In order to avoid false-negative results MRI of cranial arteries needs to be performed immediately, before or within the first days of steroid therapy. MRI is particularly useful to follow up the course of the disease and evaluate the presence of stenosis, occlusion, or aneurysm, for both LVV and PAN. In clinical practice, the degree of previously described stenosis can be evaluated as well as resolution or new damage. For future studies, a damage score is an unmet need particularly for TAK, where internal large vessel arteries are involved. PET with intravenous administration of 18F-FDG (radioactive glucose) is a useful tool of synergistic value for optimal diagnosis and monitoring disease extension, activity and treatment response in LVV (27). Areas with increased uptake of 18F-FDG is indicative for the presence of active cellular metabolism resulting from the increased glycolysis of the activated inflammatory cells. The mean sensitivity and specificity of this method is 75.9% and 93%, respectively for LVV, while the method is not useful for MVV and SVV.

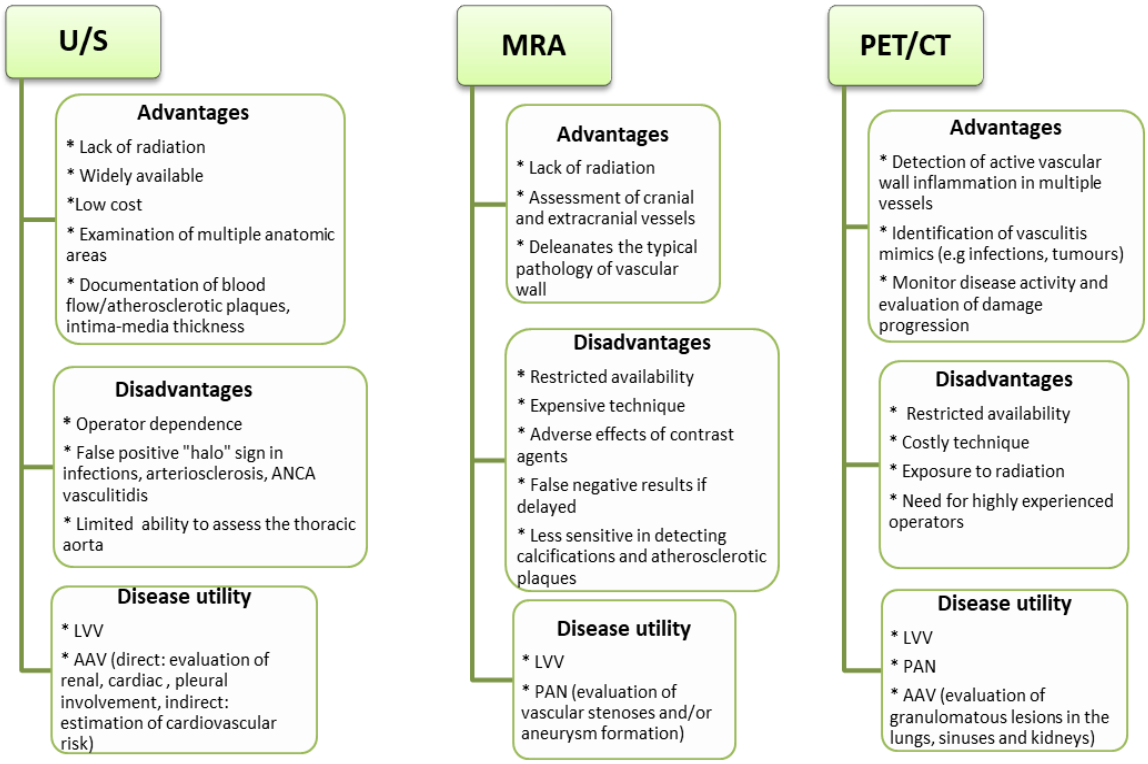

Figure 1.2 *Imaging tools for the diagnosis and monitoring of disease activity in SV.*

1.5 Treatment and prognosis

It is widely approved that the introduction of GCs has dramatically changed the natural history of systemic vasculitides over the last 30 years. Although they remain the cornerstone of therapy the intensity of the therapeutic approach fits the severity of vasculitis and ranges from low doses of corticosteroids in some cases to severe immunosuppressive treatment in others. Current treatment management usually comprises conventional immunosuppressives and immunomodulators (e.g. methotrexate, leflunomide, azathioprine, cyclophosphamide, mycophenolate mofetil) in addition to GCs right from the beginning to prevent subsequent damage and facilitate GCs tapering. With the advent of molecular immunology and the better understanding of pathogenetic mechanisms, targeted therapies involving monoclonal antibodies and small molecules binding inflammatory cytokines or inhibiting inflammatory pathways (JAK/STAT) have been investigated for the treatment of vasculitis with relative success in some and failure in others. Tocilizumab, a humanized monoclonal antibody that blocks

the receptor of IL-6, a key cytokine in the pathogenesis of LVV, is an effective treatment for and GCs sparing. The relative success of targeted therapies make much more important the clinical phenotype of SV, as well as the better understanding of the interface between active and inactive state to address questions regarding patient stratification and time of treatment administration. some open questions regarding which patients should be treated and for how long, remain unanswered. Anti-TNF agents and especially infliximab improve event-free survival, decrease disease flares and damage mainly in TAK patients. Ustekinumab, a human monoclonal antibody which blocks both IL-12 and IL-23 has been proved effective for GCA, while oral JAK1/JAK2 inhibitor has shown promising results for relapsing disease. Moderately effective has been proved for GCA abatacept, a fusion protein which prevents CD80/CD86 from binding to CD28 on the surface of T cells, resulting in failure of the costimulatory signal required for T cell activation. The presence of artery tertiary lymphoid organs in the inflamed arteries in LVV, suggest a potential role of B cells in their immunopathogenesis, thus encouraging the use of rituximab a monoclonal anti CD20 antibody which depletes B cells (28,29). Treatment of AAV is tailored according to disease stage and severity. High doses of corticosteroids and cyclophosphamide rapidly control organ or life-threatening disease but are associated with multiple short and long term adverse events. The introduction of effective medications with decreased toxicities (e.g. rituximab) has optimized AAV therapeutic management and improved survival (30). AAV are still considered to be part of a single entity because they share many clinical and histopathological features prompting the need for disease-specific studies. As such, mepolizumab, a humanized monoclonal antibody targeting IL-5 which is the major eosinophil-survival factor and has been proved effective for EGPA specific treatment (31). Current EULAR recommendations for the management of ANCA associated vasculitis treatment are summarized in ref. 30. Future studies should stratify patients according to disease phenotype, certain organ threatening clinical features and disease severity.

A constant concern for physician regarding SV is the increased frequency of disease recurrence despite adequate immunosuppressive therapy. Approximately 50-80% of GCA patients relapse during the first year and up to 40% need low dose prednisone indefinitely. Long term survival rates are 80-90% for TAK with 45% of patients developing

aortic aneurysms and nearly 40% cardiac abnormalities. The outcome of PAN depends on the presence and the extent of visceral and CNS involvement with most deaths occurring during the first year as a result of uncontrolled vasculitis, delay in diagnosis or complications of treatment. The natural history of untreated generalized AAV is that of a fatal disorder with a mean survival rate less than 1 year. Limited and mild disease may progress to widespread active disease at any time during the course in at least 10% of cases (32). Differentiation between disease activity and damage is pivotal for patients monitoring and long-term outcome. The Bermingham Vasculitis Activity Score (BVAS) is a validated tool for small and medium vessel vasculitides that records the evidence of active vasculitis arranged by organ systems but has been proved useless for assessing disease activity in LVV. Other Score systems (e.g. DEI-Tak, ITAS2010, ITAS-A), also lack credibility to depict ongoing inflammatory activity. Subsequent damage due to disease or treatment is becoming the cornerstone of long term follow up of vasculitis patients. Vasculitis damage index (VDI) is better used for LVV than for AAV. Predictors of high-level damage in these patients are older age, worse renal function, higher disease activity at presentation as well as the number of relapses.

1.6 Unmet needs in Systemic Vasculitides

The complexity of SV arises from the multi-level heterogeneity in terms of clinical phenotypes, pathogenetic mechanisms and treatment selection strategies. Despite the significant progress in the last years, there are several unmet needs (Figure 1.3), including the assessment of the different clinical phenotypes within each SV type, stratification of patients based on tissue injury and most importantly:

- **Assessment of LVV disease activity**. The lack of an accepted definition of disease activity in LVV is a major challenge in creating useful and valid outcome tools able to depict the course of the disease. Differentiation between disease activity and damage is pivotal for patients monitoring and long-term outcome. Acute phase reactants (e.g. ESR, CRP) have shown to be neither sensitive nor specific to monitor disease activity. Besides elevated in vasculitis mimics, ESR may be mildly elevated (40-50 mm/h) in 10-20% of GCA patients or normal in 33-50% of TAK

patients with active disease on arterial biopsy (33,35). The BVAS is a validated tool for small and medium vessel vasculitides that records the evidence of active vasculitis arranged by organ systems but has been proved useless for assessing disease activity in LVV (36). Other Score systems (e.g. DEI-Tak, ITAS2010, ITAS-A), also lack credibility to show inflammatory activity (37). Subsequent damage due to disease or treatment is becoming the cornerstone of long term follow up of vasculitis patients. The VDI is better used for LVV than for AAV. Predictors of high-level damage in these patients are older age, worse renal function, higher disease activity at presentation as well as the number of relapses (38). Search for, reliable, clinically useful and validated disease activity biomarkers for LVV and damage index for AAV are still ongoing.

- **Lack of biomarkers able to predict relapses**. Owing to the increased frequency of disease relapse (approximately 40-50% of vasculitis patients will recur the first year or later in the course of the disease) despite adequate immunosuppressive therapy, identification of predictors of flares is significant in optimizing therapeutic strategies, minimizing relapses, and reducing drug related side effects. The lack of widely accepted definition of disease flare not only in clinical trials but also in daily practice is a constant concern. Reappearance of clinical manifestations accompanied by elevated ESR and/or CRP is reported in only 34-41% of GCA patients, whereas other predictors (e.g. extracranial large vessel involvement, female sex, presence of anticardiolipin antibodies, hypertension, and diabetes) have not been consistently implicated. Relapses during TAK course are common but reported data on relapse frequencies are lacking (39). Moreover, the utility of the existing imaging tools (e.g. U/S, 18-FDG PET/CT) in monitoring disease activity and response to therapy by studying wall signal enhancement and other parameters remain highly controversial. **The role of ANCA level in predicting disease relapse in AAV is still under evaluation.** The transition of ANCA titers from negative to positive is considered as better predictor of disease flare than a mild rise in serum levels. Furthermore, data from two EUVAS trials (CYCLOPS and IMPROVE) showed that patients who remain ANCA positive at the time of switch from induction to remission maintenance therapy have a higher relapse rate compared with those with undetectable ANCA at switch (40).

- **Predictors of complications.** Disease phenotype and severity of expression are mainly related to morbidity and different mortality rates. In LVV multiple predictors of potential poor outcome have been suggested, but consistent predictors of ischemic complications and aneurysm formation which are the major causes of disability and mortality in these patients are lacking (35). A recent study showed that PET positive aorta of GCA patients have a Hazard Ratio around 10 to develop aneurysms compared to PET negative patients in 10 years (34). In AAV patients older age at onset, worse renal function, higher disease activity at presentation, the number of relapses and duration of GCs therapy during follow-up are proposed as predictors of level of damage (41).
- **Precise targeted treatment strategies**. The use of GCs is association with cyclophosphamide and monoclonal antibodies targeting key cytokines and cell populations with fundamental role in the pathogenesis of SV has changed the natural course of this fatal (if remain untreated) group of diseases. Although much progress has been made, there are still many unanswered questions including among others the indications and timing for targeted treatment. The recognition of different clinical phenotypes, in association with a variable genetic landscape and a vast array of different pathogenetic mechanisms suggest that approaches of Precision Medicine, might decrease morbidity and mortality rates due to drug related side effects or the disease itself.

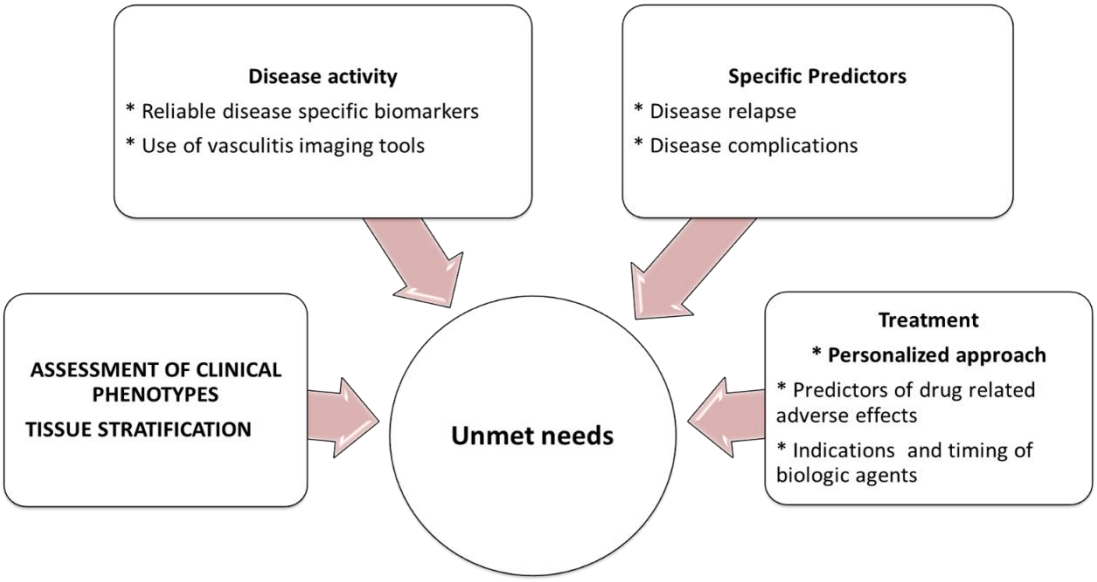

Figure 1.3 *Unmet needs in the context of Systemic Vasculitides*

1.7 Aims of the present Thesis

- Explore the presence and potential reversibility of subclinical vascular dysfunction and/or damage both in the micro- and macro-circulation in SV by evaluating four main vascular pathologies (atheromatosis, arterial stiffening, arterial remodeling / hypertrophy and pressure wave reflection impairment) in four different vascular beds (carotid, aortic, femoral and retinal) using gold-standard in clinical practice, non-invasive vascular biomarkers. The monitoring of subclinical vascular damage might provide insights on the development of these vascular pathologies in SV as well as guide the management of these patients, in similar ways as in individuals with CVD risk factors.
- Investigate the metabolome of patients with LVV at time points of activity and remission in an attempt to define new end-result biomarkers.
- Investigate the specificity of p-ANCA, the most important biomarker of AAV.
- Describe the clinical picture of cryoglobulinemic vasculitis in pSS, compared to HCV and define the biomarker(s) predicting lymphoma.

CHAPTER 2. CARDIOVASCULAR RISK IN SYSTEMIC VASCULITIS

2.1 Potential mechanisms of accelerated Atheromatosis and Arteriosclerosis in Systemic Vasculitides

Although it is widely accepted that atherosclerosis involves an ongoing inflammatory response (42), the potential mechanisms of this phenomenon are yet poorly studied in SV. Although major differences do exist, one might consider that the acceleration of arterial damage in SV shares several common mechanisms with RA, which is by far, the most extensively studied model of arterial damage in chronic inflammatory diseases (43,44). Therefore, cautious extrapolation of these mechanisms to SV seems reasonable. In brief, these mechanisms involve: the primary intramural vessel wall inflammation (Box 1, Figure 2.1); the secondary vessel wall inflammation by systemic inflammation (Box 2. Figure 2.1); SV-related drug treatment induced deleterious effects on the vessel wall (Box 3, Fig. 2.1) and the effect of classical cardiovascular disease risk factors (Box 4, Fig. 2.1)(45). Data on the prevalence of hypertension and dyslipidemia are currently lacking in SV patients, but an increased incidence compared with the general population is thought to result from the chronic use of GCs, the often presence of chronic renal damage, as well as the chronic inflammatory process per se. These general mechanisms may not only precipitate the classical inflammatory process of atheromatosis (45), but also may account for the disruption of the balance between synthesis and degradation of collagen and elastin, leading to vascular stiffening (46).

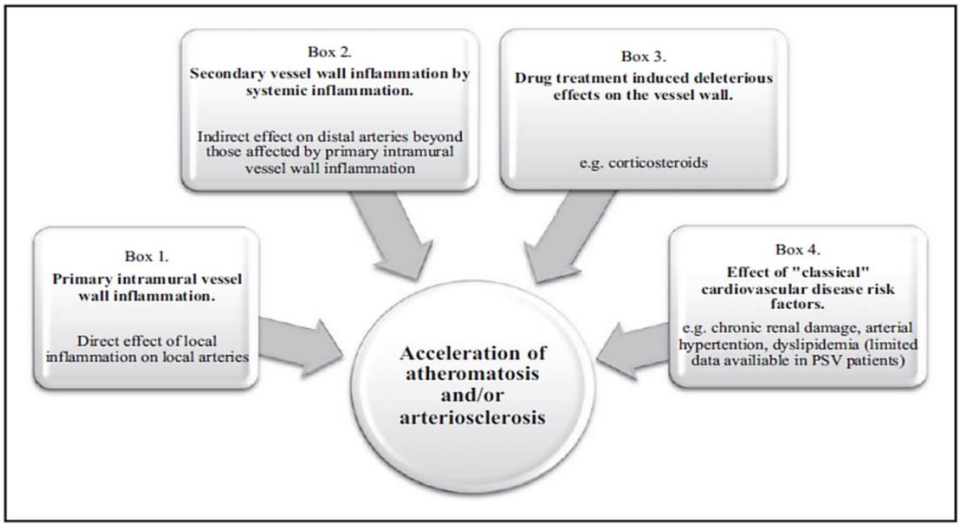

Figure 2.1 *Potential mechanisms leading to the acceleration of atheromatosis and arteriosclerosis in SV (17)*

2.2 Non-invasive biomarkers for the assessment of atheromatosis and arteriosclerosis

Atheromatosis (atheromatic plaque formation) and arteriosclerosis (reduced elasticity because of elastin fiber loss or dysfunction) represent two distinct pathways of arterial damage, which although share some common risk factors but have different damage and pathophysiological consequences. A large variety of noninvasively assessed vascular biomarkers have been developed during the past 30 years to describe these two pathways (47). The most widely applied and herein used biomarkers are described in Table 2.1. These biomarkers are used to investigate the damage of the artery and optimize cardiovascular risk stratification in clinical practice but currently cannot be used in treatment follow-up (47).

	Intima–media thickness (IMT)	Plaque presence	Carotid–femoral pulse wave velocity (cfPMV)
Measured by	Ultrasonography	Ultrasonography	Applanation tonometry (most widely applied)
Disease studied	Arterial remodeling and/or atheromatosis (at the level of the common carotid artery)	Atheromatosis	Arteriosclerosis (arterial stiffening)
Arterial bed studied	Carotid	Carotid and femoral	Aorta (thoracic and abdominal)
Recommendation to be used in clinical practice in population at intermediate cardiovascular disease risk populations	Yes	Yes	Yes

Table 2.1 Non-invasive vascular biomarkers used for the study of atheromatosis and arteriosclerosis (17).

2.2a LARGE VESSEL VASCULITIDES

Takayasu arteritis

Takayasu arteritis is a chronic inflammatory granulomatous vasculitis, manifesting mainly as a panaortitis and occurring commonly in young women between 10 and 30 years (48). Takayasu arteritis is associated with a significantly increased risk of cardiovascular complications, including cerebrovascular events, aortic aneurysm formation and ruptured and congestive heart failure. The mechanisms that link TAK with late cardiovascular complications remain to be fully elucidated (49). In Takayasu arteritis, the inflammatory process commences from the adventitia and progresses to the intima leading to segmental stenosis, occlusion, dilatation and aneurysm or

aneurysm formation. Histologically, it is characterized as a 'panarteritis' involving all layers of the arterial wall, including intimal fibrous thickening and/or typical atheromatous lesions, destruction of medial smooth muscles and elastic layers, cellular infiltration, and collagenous fibrosis in the media and thickened adventitia with cellular infiltration around vasa vasorum. Intact areas between affected areas in arteries ('skipped lesions') are usually revealed in pathological studies (50). Although the number of TAK patients is still limited, Takayasu arteritis is the most well studied SV regarding the mechanisms of atheromatosis and arteriosclerosis. Most studies have demonstrated that cIMT is significantly higher in the TAK group as compared with the control group (51,54-57). Moreover, high prevalence of atheromatic plaques is seen and cannot be explained by the traditional vascular risk factors (51,53-56). Of note, it was suggested that the abnormal cIMT might be used as a reliable marker of disease activity in TAK (sensitivity of 82% and specificity of 60%) and that it should be part of the routine evaluation of Takayasu arteritis (56,57). In daily practice, clinicians may have difficulty in making differential diagnosis between TAK-related vascular lesions and atheromatosis. Ugurlu et al. in a study of 58 patients investigated the morphologic and hemodynamic changes in the carotid arteries in TAK, along with patients with diabetes mellitus and healthy controls using Doppler USG. The study showed that carotid artery may be helpful in differentiating TAK from atherosclerosis. Diffuse homogenous increase in IMT, presence of turbulence and higher resistivity index can be considered as suggestive of Takayasu arteritis rather than atherosclerosis (52).

Increasing data also suggest that Takayasu arteritis is associated with elevated arterial stiffness in the central elastic arteries compared with controls and that arterial stiffness may persist even when the disease is quiescent. PWV was found to be significantly increased in Takayasu arteritis patients, despite the younger age and the comparable blood pressure with the control group implying that structural rather than functional vascular damage takes place in Takayasu arteritis (49,58).

Giant cell arteritis

Giant cell arteritis, the most common granulomatous SV with a predilection for large-sized and medium-sized arteries, occurs almost exclusively after the age of 50 years and affects mainly the extracranial branches of the carotid artery. It is associated with

doubled incidence of cardiovascular events and 17 times increased risk for aortic aneurysms (59-61,66). Similar to Takayasu arteritis, GCA inflammation involves all layers of the arterial wall, and the inflammatory process appears to begin in the adventitial layer at the level of vasa vasorum. Granulomatous infiltrate including giant cells is proposed to play a key role in the invasion from the adventitial side to the medial and intimal layers (63).

Several early case reports (including few patients) describe a vasculitic carotid wall thickening in GCA (63), but there are also data showing significantly lower cIMT levels compared with controls (62). A study of 41 GCA patients showed that steroid therapy has no influence on endothelial function but does significantly improves cIMT in GCA. Increasing data demonstrate that IMT measurement of temporal, facial and axillary arteries can correctly distinguish vasculitic from normal arteries in suspected GCA, thus IMT cut-off values may additionally help in the diagnosis of GCA (64). Data for wall diameters are needed for future longitudinal trials to monitor GCA treatment.

Limited data are available concerning the potential effect of GCA on arterial stiffness. A study of 49 patients showed that GCA patients have higher PWV and dilated thoracic aortas with a women preponderance compared with GCA men. Further investigation is required to evaluate the effect of severity, treatment length, disease duration and cardiovascular risk factors on aortic morphology and function (65).

2.2b MEDIUM VESSEL VASCULITIDES

Polyarteritis nodosa

Polyarteritis nodosa is an extremely rare, necrotizing vasculitis associated with aneurysmal nodules along the walls of medium-sized muscular arteries that can present initially as peripheral vascular ischemia (67,68). Poor data, involving only a few patients have been found in the literature leading to inconclusive results for the development of accelerated atheromatosis (68-71).

Kawasaki disease

Kawasaki disease is an acute medium vessel vasculitis, occurring predominantly in infants and during early childhood. The most significant complication is the

development of coronary aneurysms during the subacute phase. These aneurysms are known to cause coronary artery disease by causing thrombosis and stenosis and represent a cause of sudden death in this patient group (72,73). A review of autopsies from Kawasaki disease patients revealed that the arterial damage includes necrotizing arteritis, subacute or chronic vasculitis and luminal myofibroblastic proliferation (77).

Recent literature implicates in this, various factors like endothelial dysfunction, proatherogenic lipid profiles and arterial stiffening (73). It is unclear whether all children with Kawasaki disease have increased later cardiovascular risk. The retinal microvasculature reflects changes in the microcirculation and is associated with traditional cardiovascular risk factors and events. Larger retinal venules may reflect chronic inflammation and endothelial dysfunction and are associated with coronary artery disease in adults (74).

Carotid artery IMT is one of the most commonly used noninvasive measures of subclinical atherosclerosis in both pediatric and adult populations. There are few studies showing higher cIMT in children with KD compared with controls and others that demonstrate higher cIMT in patients of KD with coronary aneurysms (73). As cIMT and aortic IMT have been shown to be a surrogate marker of both coronary and peripheral atherosclerosis, higher cIMT and aortic IMT in children with Kawasaki disease along with proatherogenic abnormalities in lipid profile may predict a higher risk of coronary artery events in later life (73,75,76,78).

Numerous studies have demonstrated the association between KD and arteriosclerosis. Overall patients with a history of KD exhibited a high PWV relative to controls. This suggests that these patients have a subsequent tendency for increased arterial stiffness. Consequently, life-long follow-up should be advised to evaluate cardiovascular diseases caused by former KD vasculitis and age-associated factors (72,79-81).

2.2c SMALL VESSEL VASCULITIDES

ANCA-associated vasculitides

ANCA-associated vasculitides include three clinical entities: GPA, MPA and EGPA. They are necrotizing vasculitis with few or no immune deposits affecting predominantly small vessels. Compared with the general population, the risk of cardiovascular disease

(including MI, stroke, and aneurysm formation) is two-fold to four-fold higher among patients with AAV, thus exhibiting enhanced cardiovascular mortality. Patients with PR3 ANCA have a lower CVD risk than those with MPO ANCAs (82). This is based on the fact that MPO, a granule protein identified inside human atherosclerotic lesions and expressed in leucocytes (84), has been implicated both in the initiation and progression of atheromatosis (83).

Several studies, most of which concern GPA (55,85,86) have shown increased common carotid IMT in AAV patients as compared with controls (87), thus suggesting that AAV are associated with accelerated and frequently subclinical atheromatosis that cannot be explained by traditional risk factors. Furthermore, the raised levels of high sensitivity C-reactive protein, MMPs and TIMP suggest that enhanced inflammation and excessive vascular remodeling are contributing factors in the development of accelerated atheromatosis in GPA (85). Gonza´lez-Sua´rez et al. (88) in a study of 23 patients, observed an association between cIMT and internal carotid artery pulsatility index with small vessel cerebral disease pointing the possible use of carotid ultrasonography in predicting microvascular brain injury. Studies with a good number of MPA and EGPA patients were not found.

Only few data are available concerning the arterial stiffness as assessed by PWV measurement and the atherogenic index in AAV. Two studies with limited number of patients have shown increased arterial stiffness, and that arterial stiffness correlates with the degree of active inflammation in AAV patients (89,90). As far as other small vessel vasculitides (i.e. HSP and CV) are concerned, no reliable data showing accelerated atheromatosis and arteriosclerosis were found.

Behcet's Disease

Behcet's disease is a chronic, relapsing, multisystemic vasculitis involving both veins and arteries of any size (91). In addition to the classic triad (recurrent aphthous ulcers, genital ulcers, uveitis), pulmonary, gastrointestinal, nervous, and musculoskeletal manifestations may be present (93). Cardiovascular involvement (deep vein thrombosis, MI, arterial aneurysm, arterial thrombus formation) (94), occurs in 7–31% of patients and is associated with poor prognosis and increased mortality in patients with major vessel involvement (91).

Histopathologically, BD is mainly characterized by vasculitis, with prominent neutrophil and monocyte infiltration in the perivascular regions with or without fibrin deposition in the vessel wall. Endothelial dysfunction, the initial lesion in atheromatosis, as well as the intermittent inflammation, autoimmune mechanisms and drugs are thought to account for the accelerated atherosclerosis in patients with Behcet's disease (93). Of note, endothelial dysfunction in patients with Behcet's disease is modulated by the presence of corticosteroids and disease activity status. During disease relapse, corticosteroids restore endothelial dysfunction but their prolonged administration in the absence of active disease may be detrimental for the endothelium (92).

Despite previous evidence demonstrating atherosclerosis as not a prominent feature of Behcet's disease, even among patients with major organ involvement (95), a recent meta-analysis of relevant studies (96) showed that cIMT is increased in patients with Behcet's disease compared with controls. Similarly, the same meta-analysis showed that carotid plaques are three times more prevalent in patients with Behcet's disease compared with the control group, verifying the presence of accelerated subclinical atheromatosis (93,96). Another study including 50 patients showed that Behcet's disease may be associated with subtle increased cIMT, suggesting that it can be a predisposing factor for atherosclerotic arterial disease (97). Furthermore, Cure et al. (98) in a cross-sectional case–control study, showed a strong positive correlation between AIP and cIMT. These and other results (99) suggest that accelerated subclinical atherosclerosis might explain the presence of increased cardiovascular events and mortality in these patients.

Moreover, several studies showed increased arterial stiffness, assessed by carotid to femoral PWV (93). A study of 30 patients showed that PWV measurement might be more useful than cIMT in determination of vascular damage in Behcet's disease, especially in early stage of disease duration (100).

2.2d FUTURE PERSPECTIVES

Despite the fact that there are no international networks dedicated to the study of cardiovascular risk factors, the role of SV-drugs (e.g. GCs) and vascular properties in PSV, recent studies using noninvasive techniques (cIMT and PWV) have demonstrated

accelerated atheromatosis and increased arterial stiffening in these patients, thus suggesting a potential role for the increased cardiovascular events and associated mortality. Because of the limited number of SV patients and the lack of prospective data and experience on vascular biomarkers by most rheumatologists, many SV, especially PAN and SVV, remain under-studied (Table 2.2). The potential clinical value suggested by the so-far-studied vascular biomarkers would allow rheumatologists, implement optimal therapeutic strategies in the clinical practice in order to reduce the increased cardiovascular morbidity and mortality in SV. This hypothesis prompts the need for large prospective cohorts that will record cardiovascular disease risk factors and apply the methods discussed herein, as well as other noninvasive methods, in order to provide useful future guidance regarding the evaluation and restratification of cardiovascular risk, which should lead to optimization of the prognosis and treatment of SV patients.

	Evidence of increased incidence of CVD events	Evidence of accelerated atherosclerosis	Evidence of accelerated arteriosclerosis
TAK	++ (48,49)	++ (51,54,55,56,57)	++ (49,58)
GCA	++ (59,60,61,66)	++ (62,63,64)	+ (65)
PAN	+ (67,68)	NO	NO
KD	++ (72,73)	++ (73,75,76,77,78)	++ (72,79,80,81)
AAV	++ (4,83,84)	++ (55,85,86,87,88)	+ (89,90)
HSP	NO	NO	NO
CV	NO	NO	NO
BD	++ (91,94)	+++ (96,97,98,99)	++ (93,100)

Table 2.2 *Summary of data regarding the presence of atheromatosis and arteriosclerosis in SV.*

CHAPTER 3. COMMON AND RARE FORMS OF VASCULITIDES IN SJÖGREN'S SYNDROME

3.1 Introduction

Primary Sjögren's syndrome is a rather common, slowly progressive, systemic autoimmune disease, affecting mainly middle-aged women. Although the salivary and lacrimal glands are the primary targets of the disease, virtually any organ system that has epithelial structures can be affected. The precise pathogenetic mechanisms of the disease are still unknown, but it appears that both arms of the immune response, the innate and adaptive, are aberrantly activated. For the initiation of the disease, the central player appears to be the affected epithelial cell. Indeed, these cells express inappropriately molecules that can break the immune tolerance and initiate adaptive immune responses, such as human leukocyte antigen class II, intracellular autoantigens and costimulatory molecules; they are intrinsically activated, expressing functional Toll-like receptors and producing several cytokines and chemokines, able to create and restore a local inflammatory response (autoimmune epithelitis). As the autoimmune response progresses, effector mechanisms are developed. These mechanisms, that are better understood today, are the major mediators for the tissue injury and, eventually, the clinical manifestations of the disease. Indeed, the two main autoimmune phenomena, observed, when the patient is for the first time seen by a physician are two: First, the lymphocytic infiltration of the exocrine glands, consisting

predominantly of autoreactive CD4þ T lymphocytes and B lymphocytes. Second, the polyclonal autoreactive B-cell hyperactivity, leading to germinal center formation in 20–25% of patients and increased production of autoantibodies against immunoglobulins, cellular, nuclear and other antigens, as well as the activation of type-I interferon pathways (101,102). These autoimmune aberrations generate the extraglandular manifestations that are present in approximately 75% of pSS patients and are currently classified as nonspecific (arthralgias, arthritis, Raynaud's phenomenon and fatigue); periepithelial characterized by lymphocytic invasion of the epithelial tissues in parenchymal organs such as kidneys (interstitial nephritis), lungs

(small airways disease) and liver (primary biliary cirrhosis like picture); and mediated by the deposition of immune complexes due to overt B-cell hyperactivity (103). The latter include purpura, glomerulonephritis, and peripheral nerve involvement. Around 5% of pSS patients may develop NHL (104).

In pSS patients, vasculitis is one of the most clinically important extraglandular manifestations taking many clinical forms. The size and localization of the involved vessels in association with the inflammatory process, account for the wide clinical spectrum, that extends from limited cutaneous lesions to life threatening systemic disease (105,107) (Figure 3.1). By far, the most common form of systemic vasculitis in pSS is cryoglobulinaemic vasculitis. Sporadic cases of other forms of vasculitis have also been described (106-108). The clinical presentation of vasculitis in pSS is discussed below.

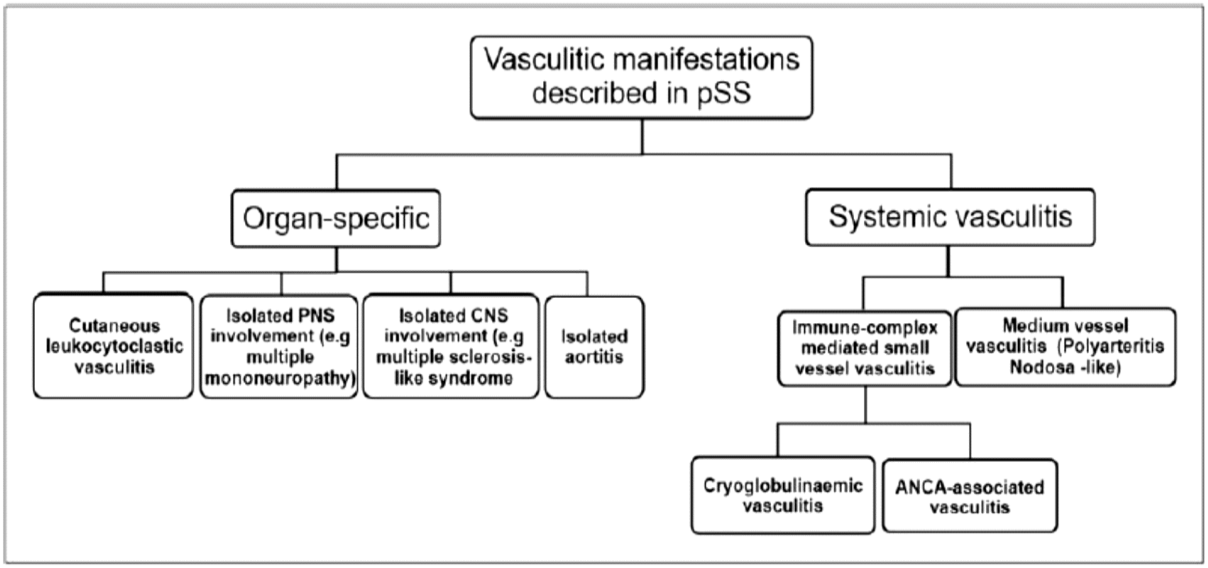

Figure 3.1 *Extraglandular manifestations of primary Sjögren's syndrome related to vasculitis (6).*

3.2 Small Vessel Vasculitis

Small vessels are the main venue of vasculitic involvement in pSS. The clinical spectrum ranges from local cutaneous disease to a multisystemic disorder with increased morbidity and mortality, especially if remains untreated. Cutaneous vasculitis generally presents as palpable, as it is the case of CV, or nonpalpable purpura, as it is

observed in urticarial vasculitis. Non-palpable hypergammaglobulinaemic purpura can also be seen frequently, particularly in patients with high levels of serum immune-globulins, located predominantly in the lower limbs. Skin vasculitis is associated with a higher prevalence of articular and renal disease as well as immunologic features ANA, RF, cryoglobulins and anti-Ro/SSA positivity (109). The histopathological examination of skin biopsies from pSS patients with skin vasculitis reveals two different SVV patterns. The leukocytoclastic pattern is the most common and it is characterized by polymorphonuclear infiltration, neutrophil fragmentation, extravasation and fibrinoid necrosis of the vessel wall. Unlike leukocytoclastic vasculitis, the lymphocytic pattern, is characterized by a different inflammatory infiltrate containing mainly lymphocytes, plasma cells and histiocytes. The vascular wall is intact, and the picture is reminiscent of a chronic autoimmune response (110,111).

Despite the fact that CNS involvement is extremely rare in pSS, inflammation of small blood vessels has been hypothesized as a potential mechanism that underlies CNS disease in some cases, in the past. These patients usually develop a multiple sclerosis-like syndrome with compatible brain lesions, cranial nerve palsies and myelitis due to inflammation of the spinal cord. It should be noted, however, that cases like these have not been described in recent cohort studies of pSS patients. Peripheral nervous system can also be affected. Distal sensorimotor, small or large fiber sensory and autonomic neuropathy have been described in patients with pSS. Vasculitic neuropathy presents as multiple mononeuropathy and accounts for about 1% of patients. Nerve biopsies commonly reveal epineural vascular inflammation with or without necrosis (112,113).

AAV has been described in sporadic cases of pSS. Guellec et al. in a recent overview of the literature described seven new and 15 previously published cases of coexisting pSS and AAV. These are most commonly p-ANCA associated vasculitis with anti-myeloperoxidase specificity, but isolated c-ANCA, anti-proteinase 3 positive vasculitis

(GPA and EGPA) were also described. Glomerulonephritis, lung, skin and PNS involvement were the most prominent, but CNS, small bowel, muscle involvement, ear chondritis and sinuses were also observed. In all cases, pSS diagnosis preceded the clinical onset of AVV (114).

3.3 Cryoglobulinaemic Vasculitis

Case report

A 56-year-old female with sicca manifestations and recurrent episodes of parotid gland enlargement the last 5 years, was admitted in the clinic for palpable purpura of the lower limbs, myalgias and painful numbness of the toes. Schirmer's test and Rose Bengal staining of the cornea were positive. Minor salivary gland biopsy disclosed round cell infiltrates with focus score 4. Immunologic and laboratory tests revealed high titer of rheumatoid factor, anti-Ro/SSA and anti-La/SSB autoantibodies, low C4 levels, hypergammaglobulinemia and leukopenia. The diagnosis of pSS was set. Subsequent laboratory evaluation revealed high titer of cryoglobulins (349.9 mg/dl) and the immunofixation demonstrated IgMk–IgG, type-II cryoglobulinemia. Nerve conduction tests were performed and disclosed sensory motor polyneuropathy. The patient was considered as having CV in the setting of pSS and was treated with steroids and anti-CD20 B-cell depletion therapy with complete resolution of her symptoms.

Among systemic autoimmune diseases, pSS is ranking first in the prevalence of CV, that is a rare systemic vasculitis, associated with the presence of serum cryoglobulins (cryoglobulinemia), affecting around 1/100 000 of the general population. It has increased morbidity and is more prevalent in the Mediterranean area. Cryoglobulins, are monoclonal immunoglobulins (type-I) or immune complexes composed mainly by either monoclonal or polyclonal IgM or IgA immunoglobulins with rheumatoid factor activity against polyclonal IgG (mixed type-II and III cryoglobulins respectively). They display the characteristic property to precipitate reversibly at temperatures less than 37 °C (Figure 3.2a). Although cryoglobulinaemia is considered as a prerequisite of the disease, not all patients with cryoglobulinemia develop clinical manifestations, suggesting that other factors are also involved in the precipitation of cryoglobulins into the affected tissues. Several causative factors have been associated with cryoglobulinemia and cryoglobulinemic vasculitis, including infections (HCV, HIV and HBV), hematologic malignancies and autoimmune diseases. The most common causes of cryoglobulinemic vasculitis are HCV infection and pSS. A systematic study to address the prevalence of CV in consecutive patients with pSS does not exist, but cohort studies suggest that it may

occur in 5–10% of pSS patients. Patients with CV or cryoglobulinemia are at high risk for B-cell origin lymphoproliferative disorders, implying that may serve as predictors of lymphoma development and therefore these patients should be closely followed-up. The pathogenetic mechanisms, mediating cryoglobulinemic vasculitis development are partially understood. However, B cells appear to be a central player, since they have been shown to clonally expand and produce monoclonal IgM rheumatoid factor that forms cold-precipitable immune complexes responsible for vasculitis. These clonal B cells display specific characteristics such as low expression of CD21 and features of functional exhaustion (CD19highCD11cþCD95þCD62Llow/neg) (115-117). For pSS, cryoglobulins are considered to rise following a chronic antigenic stimulation due to either a yet unknown viral antigen or an autoantigen, whereas the production site of cryoglobulins appears to be in the inflamed salivary epithelium (118). Significantly, the association of both HCV hepatitis and pSS with the development of B-cell lymphomas, using similar immunoglobulin VDJ (variable, diversity and joining) sequences (119) further signifies that CV and cryoglobulins are probably a common mechanism, preceding lymphoma.

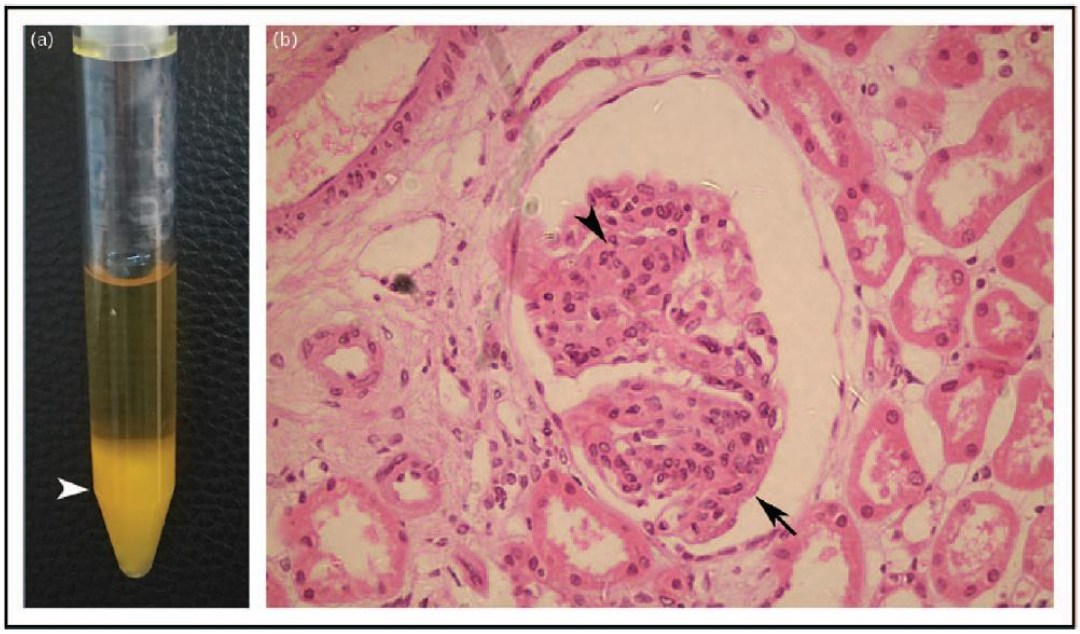

Figure 3.2 *Cryoglobulins and cryoglobulinaemic MP glomerulonephritis in pSS. (a) Serum cryoglobulins precipitated (arrowhead) after incubation at 4 8C for 7 days from a pSS patient with CV. (b)Cryoglobulinemic membranoproliferative glomerulonephritis in primary Sjögren's syndrome. Expansion of the mesangial matrix and increased cellularity of the mesangium (arrowhead) in combination with thickening and reduplication of the glomerular basement membrane producing the typical histologic picture of 'double contour' or 'tram tracking' (arrow). (Hematoxylin and eosin 400)*

(Image kindly provided by Professor Lydia Nakopoulou, First Department of Pathology, Medical School of Athens).

Cryoglobulinemic vasculitis has a wide spectrum of clinical and laboratory features (Table 3.1). The majority of CV patients in the setting of pSS complain of fatigue (80–90%) but the presenting feature of this type of vasculitis is palpable purpura (70–90%) usually at the lower limbs, leaving a brownish pigmentation as it resides. Large skin ulcers typically above the malleoli, digital necrosis, lullae and livedo racemose may also occur. A distal painful sensory or sensorimotor polyneuropathy due to vasculitis of the vasa nervosum is the most frequent neurologic manifestation of the disease (60–70%).

Clinical manifestations
Constitutional-Glandular
Fatigue
Fever
Dry eyes
Dry mouth
Dyspareunia
Lymphadenopathy
Parotid gland enlargement
Cutaneous
Purpura
Necrosis
Ulcers
Livedo reticularis
Raynaud's phenomenon
Musculoskeletal
Arthralgias/myalgias
Arthritis (symmetric nondeforming, knees and hands >elbows, ankles)
Peripheral neuropathy
Distal sensory or sensorimotor polyneuropathy
Axonopathy
Mononeuritis multiplex
CNS involvement
Multiple sclerosis-like syndrome
Cranial nerve involvement
Myelitis
Renal involvement
Glomerulonephritis (mesangial, membranoproliferative or membranous)
Proteinuria
Microscopic hematuria
Gastrointestinal
Lymphoma
Laboratory findings
ANA
Autoantibodies to Ro/La ribonucleoproteins
RF
Cryoglobulins (mixed IgMk)
Low C4
Hypergammaglobulinaemia
Leukopenia

Axonopathy and mononeuritis multiplex have been also described (112,120). Renal disease occurs in 20–35% of CV patients and ranges from asymptomatic to acute renal failure. Proteinuria is the most common clinical expression with MP glomerulonephritis (Figure 3.2b) presenting subendothelial deposits of cryoglobulins being the most common histological finding (80%) (121). Type-II cryoglobulinemia as well as high serum cryoglobulin concentration have been proposed as risk factors for renal disease independently to vasculitic involvement (122).

Table 3.1 *Organ involvement and laboratory findings related to CV in pSS patients.*

3.4 Medium Vessel Vasculitis

Unlike SVV, inflammation of medium vessels is very infrequent in pSS occurring in less than 5% of pSS patients with vasculitic involvement and, thus, only a few case reports were found in the literature. Acute necrotizing vasculitis resembling PAN in pSS patients was first described by Tsokos et al. in 1987. The disease was predominantly localized in internal organs and associated with life-threatening symptoms. Histologically, the vascular wall exhibited heavy infiltration with acute and to a lesser degree, with chronic inflammatory cells. Fibrinoid necrosis was present, but the patients lacked the characteristic aneurysmal formation (111). Similarly, another group found that two of 52 pSS patients had vasculitis of medium sized arteries. One had coexistent PAN and one necrotizing vasculitis of the pancreatic and mesenteric arteries (109). Another form of vasculitic involvement seen in pSS is endarteritis obliterans, a noninflammatory obstructive vasculitis affecting medium-sized vessels. This condition presents with fibrous thickening of the intima, leading to variable lumen stenosis with recanalization and thus, impairment of blood flow, causing ischemia/infarction of the dependent tissues. Residual mononuclear cells may be seen in the adventitia suggesting that this type of vasculitis may in fact represent the healing stage of preexisting acute vasculitis (111).

3.5 Large Vessel Vasculitis

Large vessel vasculitis represents a heterogeneous group of rare diseases characterized by intramural inflammation of the aorta, its major branches and extremity arteries. So far, data suggest that the inflammatory invasion, stimulated by an unknown trigger, commences from the adventitia at the level of vasa vasorum and progresses to the intima leading to segmental stenosis, occlusion and aneurysm formation. Several molecular mechanisms, also involved in the pathogenesis of pSS, including matrix metalloproteinase activation, c-Jun N-terminal kinase, nuclear factor kappa-light chain-enhancer of activated B cells and transforming growth factor beta signaling pathways are implicated in the initiation and progression of aortic aneurysms and aortic dissection (123). Significantly, while LVV are mainly regarded as T-cell

mediated diseases, the immunohistochemical analysis of aortic tissue samples of LVV patients, who underwent aortic aneurysm surgery, revealed massive B-cell infiltrates organized into ectopic germinal centers, within the affected arterial wall (124). In pSS patients, LVV is rare and only a few cases have been reported in the literature. The first case of inflammatory abdominal aortic aneurysm in pSS patient with positive anti-Ro and anti-La autoantibodies was reported by Ghinoi et al. (125), whereas more recently Heper et al. (126) described a case of seronegative pSS patient with pleural and pericardial effusions, ascites and ascending aortic aneurysm with increased uptake of 18-Fluorodeoxyglucose (FDG) in FDG–PET. A recent retrospective cohort study of 10941 Chinese Sjögren's syndrome patients showed that pSS patients exhibit not only increased prevalence of cardiovascular risk factors, including hypertension, hypertriglyceridemia and early atheromatosis as previously shown (127), but also increased risk of aortic aneurysm and aortic dissection (128). This clinical observation has not been described in non-Asian patients with pSS, and remains an open question, that can be addressed using noninvasive methods of vascular inflammation, including vascular ultrasound or PET–FDG (17). The common signaling pathways and the emerging role of B cell hyperreactivity in aortic aneurysms, could suggest that chronic aortitis might be a manifestation of a systemic autoimmune process rather than an aberrant local reaction to atherosclerosis. Cerebral LVV has also been described in strongly anti-Ro and anti-La positive pSS patients, but an etiologic linkage between the two diseases cannot be supported yet (129).

3.6 Prognosis of vasculitis in pSS

GEMESS Study Group examined the clinical features in 1010 Spanish pSS patients and found that increased frequency of vasculitis was associated with younger age (disease onset <35 years), long-term (>10 years) duration, as well as positive ANA, rheumatoid factor and anti-Ro/La (130). Type-II cryoglobulinemia is present in approximately 5–20% of pSS patients and has been associated with high prevalence of extraglandular disease, increased levels of rheumatoid factor, anti-Ro/SSA and low serum C4 (131). Tzioufas et al. (132) were the first to demonstrate that mixed monoclonal cryoglobulinemia is a

predictive factor for lymphoma development. Three years later Voulgarelis et al. found that NHL patients exhibit increased prevalence of skin vasculitis (11%). Most of these patients had increased levels of rheumatoid factor and cryoglobulins and low C4 serum levels (133). CV is a major factor of increased morbidity in pSS. Despite the fact that most patients experience a slowly progressive and benign course, 35% present with moderate to severe disease course involving internal organs. The 10-year survival rate in mixed cryoglobulinemic vasculitis is 56%. The worse prognosis is conferred by glomerulonephritis especially in older aged (≥60 years), male patients. Higher serum creatinine levels and increased proteinuria at CV diagnosis predispose to kidney failure and death (116,134,135). The correlation between CV and lymphoma development that has been discussed above, has an additive effect in the mortality of pSS and CV patients. Symptomatic lymphomas are reported in 5–20% of CV patients within 10 years of diagnosis, with B-cell lymphomas of the mucosa associated lymphoid tissue type being the most common. Immunosuppression due to chronic immunosuppressive therapy accounts for serious infections, but this constitutes a less frequent cause of death in these patients (136-138).

3.7 Treatment of pSS related vasculitis

As previously reported systemic vasculitis is a potentially life-threatening disease especially, if remains untreated. For immune-complex mediated systemic vasculitis (AAV and CV) corticosteroids are the gold-standard of initial management especially in patients with severe multiorgan involvement. Steroids should be tapered quickly and there is no rule for chronic therapy. Other immunosuppressive agents including cyclophosphamide, azathioprine and methotrexate are also used as induction or maintenance therapy especially in patients with renal or PNS involvement with variable response. It should be noted, however, that chronic exposure of patients with pSS to alkylating agents may increase the potency of lymphomagenesis.

Anti-CD20 treatment (Rituximab), targeting B cells, has shown a very good efficacy in treating mixed CV (aiming to limit the production of cryoglobulins) and AAV and should be considered in all patients with severe to moderate disease (116,120,139,140). In

patients with hyperviscosity syndrome due to increased concentration of cryoglobulins, as well as rapidly glomerulonephritis (mainly seen in AAV) plasma exchange can serve as an additional therapeutic option (140,142). Potential therapies for refractory CV cases are mycophenolate mofetil and belimumab while tumor necrosis factor inhibitors have been proved ineffective (143). Colchicine has been reported to have favorable effect on purpura and leg ulcers (142). High dose of steroids are the cornerstone of LVV therapy. Methotrexate, mycophenolate mofetil, azathioprine and hydroxychloroquine have been used as steroid sparing agents. For refractory disease, steroid dependent or resistant cases the anti-IL6R biologic agent (Tocilizumab) is recommended as an effective alternative. Anti-TNF agents can be also considered in recalcitrant disease despite the conventional therapy (145).

3.8 Future perspectives

Vasculitis is a severe extraglandular manifestation of pSS contributing to the increased morbidity and mortality of the disease. Mixed cryoglobulinemia, glomerulonephritis and purpura are associated with a four-fold increased risk of NHL development. The properties of the disease can serve as an excellent model to get further insights into the pathogenetic mechanisms underlying, autoreactive B cells, autoantibody production and the transition from benign B-cell polyclonality to malignant lymphoma. Further studies aiming to characterize B cells and mechanisms mediating their activation/differentiation/transition; discover and validate biomarkers predicting the development of CV; and the harmonization and stratification of CV patients, are anticipated to provide relevant insights into autoimmunity and hematologic malignancies and reveal novel therapeutic targets.

PART II. CLINICAL AND EXPERIMENTAL DATA

CHAPTER 4. NON-INVASIVE VASCULAR BIOMARKERS IDENTIFY EARLY VASCULAR DAMAGE AND GUIDE THE MANAGEMENT OF CARDIOVASCULAR RISK FACTORS IN PSV.

4.1 Objectives

Primary systemic vasculitides constitute an heterogenous group of rare, chronic and often recurrent systemic autoimmune/autoinflammatory diseases, that are subdivided, in terms of nomenclature classification according to the size of the predominantly affected vessels, to large, medium and small vessel vasculitis (1). Their hallmark is the multi-level heterogeneity regarding the clinical phenotype, histologic patterns, pathogenetic mechanisms and treatment selection strategies (18). In PSV, the vascular damage characterized mainly by transmural inflammatory degeneration of the vascular wall, implicates various disease-specific mechanisms including cellular immunity, immune complex formation and presence of antineutrophil cytoplasmic antibodies (ANCA) (146).

The acceleration of three classical types of arterial damage (inappropriate arterial remodeling, atheromatosis and arteriosclerosis), affecting both the micro- and macro-circulation, has also been proposed at least in some PSV types (17). This is attributed to the interplay between tissue and systemic inflammation, immunosuppressive therapy, and common cardiovascular disease-(CVD) risk factors (147) and may contribute to the observed increased morbidity and mortality of PSV patients (148,149), thus raising challenges and potential opportunities for the individualization and optimization of PSV management. Based on easy to use, non-invasive vascular indices, the monitoring of subclinical vascular damage (arterial hypertrophy / remodeling, atheromatosis and arterial stiffening), not only provides insights on the development of these vascular pathologies in PSV, but might be also used to guide treatment, in similar ways as in individuals with CVD risk factors (47,150).

So far, the presence of accelerated atheromatosis, arteriosclerosis and inappropriate arterial remodeling have been partly described in PSV. Stronger data exist for TAK, KD and BD, and weaker for GCA and AVV (5,83,84,93,151-154). Most importantly these data derive from small cross-sectional studies, with methodological drawbacks, including evaluation of isolated arterial properties in single arterial beds and lack of

follow-up data investigating the potential reversibility of arterial dysfunction or damage according to the inflammatory bulk. Of note, alterations of the retinal microcirculation and pressure wave reflections, parameters strongly associated with CVD mortality (150,155), have not been studied in PSV.

The aim of the present study is to explore the presence and potential reversibility of subclinical vascular dysfunction and/or damage both in the micro- and macro-circulation in PSV by evaluating four main vascular pathologies (atheromatosis, arterial stiffening, arterial remodeling/hypertrophy and pressure wave reflection-(PWR) impairment) in four different vascular beds (carotid, aortic, femoral and retinal) using gold-standard in clinical practice, non-invasive vascular biomarkers.

4.2 Study population and design

This is a prospective, matched 1:1, case-control study of consecutive, consenting PSV patients recruited from the rheumatology outpatient clinic of the department of Pathophysiology, between September 2017 and December 2021. All patients were classified according to CHCC 2012 nomenclature (1), and the respective per disease international classification criteria (156-162). Multiple control groups were used. First, to address the hypothesis that PSV patients exhibit subclinical vascular damage during active disease, apparently healthy individuals free of inflammatory burden, established CVD and of any other chronic disease, that were referred to the Cardiovascular Research Laboratory of Laiko General Hospital for CVD risk assessment, were included in the analysis-(non-inflammatory control group-NIC). Second, to address the potential effect of high inflammatory burden on arterial function and structure, patients with PMR without underlying vasculitis and patients with RA were used as disease controls. All PSV, PMR and RA patients were hospitalized or attaining the corresponding outpatient clinics of Laiko General Hospital.

Matching was performed according to age, sex, presence of CVD history, BMI, smoking status, AH including MAP during vascular measurements), dyslipidemia, HR, DM as well as treatment related to CVD risk factors as follows: (i) all (with active and inactive chronic disease) PSV patients were matched at 1:1 ratio with NIC and RA-controls, and (ii) GCA and SVV patients were matched 1:1 and compared with PMR and RA-controls.

For some PSV patients, vascular assessment of non-invasive vascular biomarkers was performed at two time points: at study entry and upon remission (around 6 months after baseline).

Demographic characteristics, CVD risk factors and associated medication, as well as detailed clinical and laboratory data and disease related immunosuppressive therapy were recorded at study entry and follow-up visits. In selected cases life- or organ-threatening disease of some patients at study entry imposed the urgent and short-term (≤ 7 days) initiation of immunosuppressive therapy prior to vascular assessment. The global study design is presented in Figure 4.1 and has been partly published previously (23). The study was approved by the Ethics Committee of School of Medicine, National and Kapodistrian University of Athens, Greece (protocol no: 1718016656). All participants provided written informed consent for the collection and use of all data, whereas the general data protection regulations and the Helsinki Declaration were routinely followed. The study was partially funded by the Hellenic Rheumatology Society.

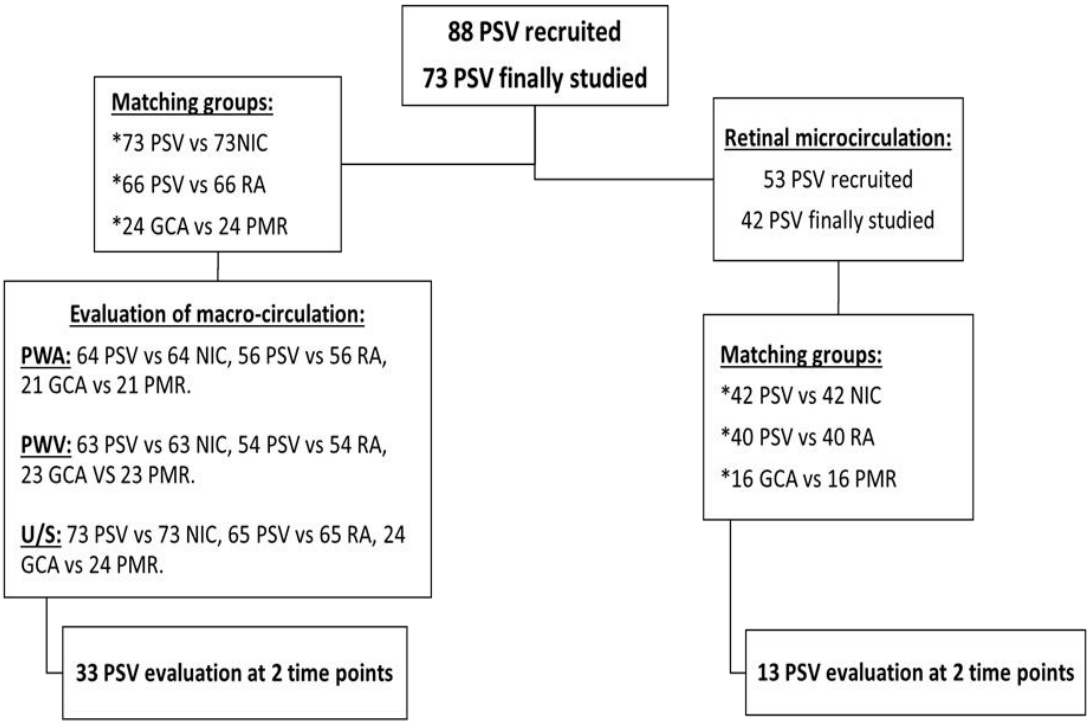

Figure 4.1 *Global study design.*

Exclusion criteria

Exclusion criteria for all participants (PSV group and the control groups) were: i) age below 18 years, ii) other underlying systemic autoimmune/autoinflammatory disease, iii) history of neoplastic disease for at least 10 years prior to study recruitment, iv) active infectious disease or hospitalization for infectious disease for at least 6 months before study entry, v) secondary hypertension, vi) absence of sinus rhythm during laboratory testing, vii) modifications in CVD medication in the past month, and viii) unwillingness/inability to adhere to study protocol.

Definitions of CVD risk factors and Disease Activity

Arterial hypertension was defined as the use of antihypertensive drugs and/or office blood pressure measurement higher than 139 and/or 89 mmHg, dyslipidemia as the use of lipid-modifying drugs and/or LDL level higher than 160 mg/dl, DM was defined as glucose higher than 126 mg/dl or HbA1c higher than 6.5% and/or glucose-lowering treatment and ex-smoking discontinuation for more than 6 months. Due to the lack of a common disease activity scoring system for all PSV, PMR and RA patients, active disease was defined as the presence of clinical symptoms and/or increased acute phase reactants (erythrocyte sedimentation rate, ESR >20 mm/h and c-reactive protein, CRP >5 mg/dl) while inactivity none of the above. 18FDG Positron emission tomography with computed tomography, performed by a highly experienced nuclear physician using the same protocol, was used at study entry to exclude subclinical vasculitis in PMR patients and assess the extent of vascular involvement in LVV and MVV patients.

4.3 Protocols for the assessment of non-invasive vascular biomarkers

All participants followed a common preparation protocol, including refraining from food, vasoactive substances, or medication at least 3 hours before the vascular assessment. All tests were performed after at least 10 minutes of rest in the supine position by the same well-trained operator.

Arterial stiffness

Arterial (aortic) stiffness was assessed using carotid to femoral PWV, a common, validated applanation tonometric method (Sphygmocor device; Atcor Medical, Sydney, Australia). Two different pulse waveforms were obtained, one at the right common carotid artery and the second at the right femoral artery using a high-fidelity handheld tonometer (SPT-301, Millar Instruments Inc. Houston, TX, USA). Carotid and femoral pulse wave sequences were synchronized by using the simultaneously recorded ECG. Pulse transit time from the carotid to the femoral artery was estimated by the tangential method, implemented by the SphygmoCor device. The PWV was calculated by the ratio of the distance traveled by the recorded pressure waves to the pulse transit time (in m/sec). The distance was determined as the length from the suprasternal notch to femoral artery minus the length from carotid artery to the suprasternal notch. At least two repeated measurements of carotid to femoral PWV were performed and their average value was used in the analysis in accordance with previous recommendations (47). Arteriosclerosis was defined as PWV values greater than 10 m/s.

Brachial / aortic BP and Pressure Wave Reflections (AIx)

Office brachial BP measurements were performed with a validated automated oscillometric device (Microlife WatchBP Office, Microlife AG, Widnau, Switzerland). Three consecutive measurements (with 1 minute interval) were obtained, and the average was used in the analysis. Office aortic pressure waveforms was assessed through applanation tonometry of the radial artery using a high-fidelity handheld tonometer (SPT-301, Millar Instruments Inc. Houston, TX, USA) and use of validated generalized transfer function (Sphygmocor, AtCor Medical, Sydney, Australia). Arterial waveforms at the radial artery were recorded during an 8 sec period and the pulse was calibrated by the preceding brachial BP measurements. The peripheral pressure waveform was converted into a central aortic waveform using an incorporated validated generalized transfer function. Augmentation pressure was determined using SphygmoCor software and identified as the difference between aortic pulse pressure (PPc) and the first systolic shoulder of the aortic pulse wave. Aortic AIx was determined as AP/PPc, expressed as a percentage and normalized for the heart rate of 75 bpm (AIx75). At least two recordings were performed, and the average was used. The

quality control of pressure waveforms provided by the Sphygmocor software was based on beat-to-beat repeatability, sharp upstroke, definite sharp incisura, and near exponential pressure decay in late diastole (163).

Atheromatosis and Arterial hypertrophy

Carotid and femoral ultrasonography was performed for the assessment of IMT and presence of atheromatic plaques (B mode high frequency ultrasonography: Logiq V5 Expert; GE Healthcare, Fairfield, Connecticut, USA). Specifically, IMT was measured in adjacent to any plaque (if present) at the far wall of the common carotid artery in end-diastole of the cardiac cycle using semi-authomatic software (165). Carotid hypertrophy was defined as left common carotid artery IMT > 0.9 mm. Atheromatic plaque was defined as local increase of the IMT of >50% compared to the adjacent vessel wall or a bulging to the lumen IMT>1.5 mm. Carotid atheromatosis was defined as any plaque presence at the (left or right) common carotid arteries or carotid bulbs and femoral atheromatosis as presence of plaque at the (left or right) femoral arteries (47).

Retinal vessel calibers evaluation

Both eyes of each participant were photographed with a 45° digital, non-mydriatic retinal camera (Topcon TRCNW8, Tokyo, Japan) after 5 min of adaptation in the dark using a validated method (165). Retinal images were centered on the optic disc and followed by quantitative retinal grading. All tests were conducted by a well-trained in retinal microcirculation physician (EKA) blinded to the clinical data. For each photograph, the calibers of retinal arterioles and venules passing through a zone extending from 0.5 to 1.0 disc diameters from the optic disc margin were measured and analyzed using a Static Retinal Vessel Analyzer (SVA-T and Vesselmap 2 software, Visualis, Imedos Systems UG, Jena, Germany) (165). These measurements were then summarized using previously described formulas (166) to compute the central retinal arteriolar equivalent (CRAE) and the central retinal venular equivalent (CRVE), representing the average internal caliber of retinal arterioles and venules, respectively. In addition, the arteriolar to venular ratio (AVR) was computed as the quotient of CRAE over CRVE. The intra-observer reproducibility of retinal vascular measurements was excellent (intraclass correlation coefficients >0.93).

4.4 Statistical analysis

Statistical analysis was performed using the STATA 13 software package and the SPSS statistical package (IBM, version 21.0). Statistical significance was defined as p-value ≤0.05. For description purposes frequencies distributions and mean plus SD, were used for all quantitative data, while frequencies were used for qualitative ones. Multivariable linear regression with further adjustment for MAP, HR during the vascular examination was performed to compare left carotid cIMT, AIx and PWV between patients and their respective matched controls; AIx75 was adjusted only for MAP. To compare between patients and controls for the presence of atheromatic plaques only univariable logistic regression was performed, since all patients were matched. Further analysis over the correlation of PWV, IMT, AIx and AIx75, as well as retina vessel calibers with CRP, ESR and their fluctuations after the follow up period among the patients were conducted using Pearson correlation. For the presence of atheromatic plaques univariate logistic regression was used.

4.5 Results

4.5a Patients' characteristics

Seventy-three PSV patients were included in the study population. Of those, 42 (57.5%) were diagnosed with a type of LVV, 4(5.5%) with MVV and 27(37%) with SVV. The male to female ratio of the total cohort was approximately 1:3 and the mean age at study entry 63±16 years. Active disease at study entry was observed in 65 (89%) of patients. A detailed description of all patients, stratified per PSV type and their matched controls characteristics, at study entry, are presented in Tables 4.1-4.5 respectively. In the retina study, retinal vessel calibers were evaluated in 42 patients with PSV fulfilling the study selection criteria, of whom 21(50%) had LVV, 3 (7.1%) MVV and 18 (42.9%) SVV. Active disease was detected in 29(69.1%) of patients. The vast majority of all participants were females and the mean age at study entry was 59.4±12.1 years. A descriptive analysis of the features of all participants are shown in Tables 4.6 and 4.7.

	All PSV (n=73)	NIC (n=73)	Active PSV (n=65)	NIC (n=65)	Inactive PSV (n=8)	NIC (n=8)
Age (mean±SD, years)	63±16	62.8±15.6	63±16	62.8±15.6	63±17	62.8±16.8
Gender (female %) (n)	72.6(53)	72.6(53)	73.9(48)	73.9(48)	62.5(5)	62.5(5)
Disease type (%) (n)						
LVV & MVV	63(46)	-	63.1(41)	-	62.5(5)	-
GCA	49.3(36)		50.8(33)		37.5(3)	
TAK	6.8(5)		4.6(3)		25(2)	
IgG4 aortitis	1.4(1)		1.5(1)		0(0)	
PAN	5.5(4)		6.2(4)		0(0)	
SVV	37(27)		36.9(24)		37.5(3)	
GPA	12.3(9)		12.3(8)		12.5(1)	
MPA	12.3(9)		12.3(8)		12.5(1)	
EGPA	1.4(1)		1.5(1)		0(0)	
HSP	4.1(3)		3.1(2)		12.5(1)	
SKIN	5.5(4)		6.2(4)		0(0)	
CV	1.4(1)		1.5(1)		0(0)	
Disease course (%) (n)						
Newly diagnosed	65.8(48)	-	29.2(19)	-	0(0)	-
Relapse	26(19)		6.2(4)		37.5(3)	
Chronic	8.2(6)		1.5(1)		62.5(5)	
Active disease (%) (n)	89(65)		100(65)		0(0)	
Clinical	0(0)		0(0)		0(0)	
Laboratory	16.4(12)		1.5(1)		0(0)	
Both	72.6(53)		35.4(23)		0(0)	
CVD (%) (n)	9.6(7)	8.2(6)	10.8(7)	9.2(6)	0(0)	0(0)
CVD Risk factors						
BMI (mean±SD)	26.9±6.1(67)	27.3±4.2(73)	26.8± 6.3(60)	27.4±4.1(65)	27±4.4(7)	26.5±4.9(8)
Smoking (%) (n)						
Current	24.7(18)	12.3(9)	26.2(17)	7.7(5)	12.5(1)	12.5(1)
Ex-smokers	19.2(14)	(18)	16.9(11)	24.6(16)	37.5(3)	25(2)
AH (%) (n)	60.3(44)	67.1(49)	64.6(42)	69.2(45)	25(2)	50(4)
SBP (mean±SD)	127.5±19.3(72)	136±20(73)	127.2±19.9(64)	137±19(65)	129.7±14.2(8)	136±29(8)
DBP (mean±SD)	72.3±10.5(72)	79±11(73)	72.3±10.8(64)	79±10(65)	71.9±8.7(8)	79±18(8)
PMean (mean±SD)	92.6±13(71)	91.1±12.2(73)	92.2±13.6(63)	91.1±10.8(65)	95.5±7.1(8)	90.9±21.4(8)
HR	72±11.6(72)	69±11(73)	72.1±11.4(64)	69±11(65)	71.5±15(8)	70±9(8)
Dyslipidemia (%) (n)	42.5(31)	47.9(35)	38.5(25)	46.2(30)	75(6)	62.5(5)
TCHOL (mean±SD)	186.3±58.8(67)	211±39(69)	182.6±57.7(60)	210±37(61)	218.4±62.8(7)	215.8±54.2(8)
LDL (mean±SD)	117.5±43.6(60)	126±34(68)	116.1±42.9(53)	127±34(60)	128.1±51(7)	124.8±37.7(8)
HDL (mean±SD)	51.2±17.7(61)	60±16(67)	50±18.1(54)	59±17(59)	60.1±11.9(7)	61.8±16.2(8)
TG	129.1±57.4(65)	123±77(69)	124.5±48.7(58)	121±59(61)	167±103.6(7)	139.6±163.1(8)
Diabetes (%) (n)	9.6(7)	12.3(9)	10.8(7)	12.3(8)	0(0)	12.5(1)
Glu (mean±SD)	99.3±26.3(72)	98±18(66)	100.8±27.1(64)	98±19(58)	0(0)	12.5(1)
Laboratory features						
ESR (>20 mm/h) (%) (n)	78.1(57/73))	25.8(8/31)	87.7(57/65)	27.6(8/29)	0(0/8)	0(0/2)
ESR (mean±SD,mm/h) (n)	53.3±34.1(73)	17±11(31)	58.6±32.3(65)	18±11(29)	9.9±3.7(8)	8.5±4.9(2)
CRP (>5 mg/L) (%) (n)	73.6(53/72)	8.7(2/23)	82.8(53/64)	9.5(2/21)	0(0/8)	0(0/2)
CRP (mean±SD)	41.8±57.3(72)	2.5±1.6(23)	46.9±58.8(64)	2.6±1.7(21)	1.4±0.9(8)	1.6±0.6(2)
Comorbidities related treatment (%) (n)						
Blood Pressure lowering drugs	54.8(40)	52.1(38)	58.5(38)	52.3(34)	25(2)	50(4)
Lipid modifying	32.9(24)	28.8(21)	27.7(18)	27.7(18)	75(6)	37.5(3)
Glucose lowering drugs	8.2(6)	12.3(9)	9.2(6)	12.3(8)	0(0)	12.5(1)
Disease related treatment (%) (n)						
Glycocorticosteroids	74(54)	0(0)	75.4(49)	0(0)	62.5(5)	0(0)
Immunosuppressives	41.1(30)	0(0)	36.9(24)	0(0)	75(6)	0(0)

Table 4.1 *Baseline - at study entry - characteristics of all patients with active and inactive disease as compared to their matched NI controls.*

	All PSV (n=66)	RA Controls (n=66)	Active PSV (n=58)	RA Controls (n=58)	Inactive PSV (n=8)	RA Controls (n=8)
Age (mean±SD, years)	63±15	62±15	62±15		63±17	61±18
Gender (female %) (n)	74.2(49)	74.2(49)	75.9(44)	75.9(44)	62.5(5)	62.5(5)
Disease type (%) (n)						
LVV & MVV	65.2(43)	-	65.5(38)	-	62.5(5)	-
GCA	50(33)		51.7(30)		37.5(3)	
TAK	7.6(5)		5.2(3)		25(2)	
IgG4 aortitis	1.5(1)		1.7(1)		0(0)	
PAN	6.1(4)		6.9(4)		0(0)	
SVV	34.9(23)		34.5(20)		37.5(3)	
GPA	13.6(9)		13.8(8)		12.5(1)	
MPA	10.6(7)		10.3(6)		12.5(1)	
EGPA	1.5(1)		1.7(1)		0(0)	
HSP	3.0(2)		1.7(1)		12.5(1)	
SKIN	4.6(3)		5.2(3)		0(0)	
CV	1.5(1)		1.7(1)		0(0)	
Disease course (%) (n)						
Newly diagnosed	63.6(42)	-	72.4(42)	-	0(0)	-
Relapse	27.3(18)		25.9(15)		37.5(3)	
Chronic	9.1(6)		1.7(1)		62.5(5)	
CVD (%) (n)	9.1(6)	10.6(7)	10.3(6)	12.1(7)	0(0)	0(0)
CVD Risk factors						
BMI (mean±SD)	27.2±(61)	27.8±5.1(66)	27.2±6.5(54)	27.6±5.0(58)	27±4.4(7)	29.2±6.5(8)
Smoking (%) (n)						
Current	27.3(18)	27.3(18)	29.3(17)	25.9(15)	12.5(1)	37.5(3)
Ex-smokers	18.2(12)	27.3(18)	15.5(9)	29.3(17)	37.5(3)	12.5(1)
AH (%) (n)	59.1(39)	57.6(38)	63.8(37)	56.9(33)	25(2)	62.5(5)
SBP (mean±SD)	127.3±19.3(65)	133±21(65)	127±20(57)	135±22(57)	129.8±14.2(8)	124±11(8)
DBP (mean±SD)	72.4±10.6(65)	78±14(65)	72.4±10.9(57)	78±15(57)	71.9±8.7(8)	75±5(8)
PMean (mean±SD)	92.9±13(64)	96.6±12.7(62)	92.5±13.7(56)	97±13.2(55)	95.5±7.1(8)	93.6±6.4(7)
HR	72.7±11.3(65)	73±11(63)	72.8±10.8(57)	74±11(56)	71.5±15(8)	69±14(7)
Dyslipidemia (%) (n)	42.4(28)	36.4(24)	37.9(22)	34.5(20)	75(6)	50(4)
TCHOL (mean±SD)	192.2±59(60)	204±36(60)	188.7±58.2(53)	210±33(53)	218.4±62.8(7)	161±22(7)
LDL (mean±SD)	120.5±44.1(55)	126±33(54)	119.4±43.5(48)	130±31(49)	128.1±51(7)	85±15(5)
HDL (mean±SD)	51.3±17.5(56)	59±16(53)	50±17.8(49)	59±16(48)	60.1±11.9(7)	55±18(5)
TG (mean±SD)	133.8±57.2(58)	111±44(55)	129.3±47.6(51)	113±46(48)	167±103.6(7)	97±32(7)
Diabetes (%) (n)	9.1(6)	9.1(6)	10.3(6)	8.6(5)	0(0)	12.5(1)
Glu	98.9±24.3(65)	93±14(62)	100.6±25.1(57)	92±14(55)	87.4±13.9(8)	101±14(7)
Laboratory features						
ESR (>20 mm/h) (%) (n)	78.8(52/66)	81.8(54/66)	89.7(52/58)	93.1(54/58)	0(0/8)	0(0/8)
ESR (mean±SD,mm/h) (n)	53.9±34.4(66)	33±19(66)	60±32.3(58)	37±17(58)	9.9±3.7(8)	9±5(8)
CRP (>5 mg/L) (%) (n)	70.8(46/65)	54.8(34/62)	80.7(46/57)	63(34/54)	0(0/8)	0(0/8)
CRP (mean±SD)	42±57.7(65)	10.2±10.9(62)	47.7±59.4(57)	11.4±11.3(54)	1.4±0.9(8)	2.1±1.1(8)
Comorbidities related treatment (%) (n)						
Blood Pressure lowering drugs	54.5(36)	47(31)	58.6(34)	44.8(26)	25(2)	62.5(5)
Lipid modifying	34.9(23)	24.2(16)	29.3(17)	20.7(12)	75(6)	50(4)
Glucose lowering drugs	7.6(5)	4.5(3)	8.6(5)	5.2(3)	0(0)	0(0)
Disease related treatment (%) (n)						
Glycocorticosteroids	72.7(48)	68.7(46)	74.1(43)	69(40)	62.5(5)	75(6)
Immunosuppressives	43.9(29)	83.3(55)	39.7(23)	81(47)	75(6)	100(8)

Table 4.2 *Baseline - at study entry - characteristics of patients per disease group as compared to their matched RA controls.*

	LVV & MVV (n=46)	*NIC (n=46)*		*SVV (n=27)*	*NIC (n=27)*
Age (mean±SD, years)	67±13	66.4±12.9		57±18	57±18.1
Gender (female %) (n)	82.6(38)	82.6(38)		55.6(15)	55.6(15)
Disease type (%) (n)		-	GPA	33.3(9)	-
GCA	78.3(36)		MPA	33.3(9)	
TAK	10.9(5)		EGPA	3.7(1)	
IgG4 aortitis	2.2(1)		CV	3.7(1)	
PAN	8.7(4)		HSP	11.1(3)	
Disease course (%) (n)			SKIN	14.8(4)	
Newly diagnosed	63(29)			70.4(19)	
Relapse	30.4(14)			18.5(5)	
Chronic	6.5(3)			11.1(3)	
Active disease (%) (n)	89.1(41)			88.9(24)	
Clinical	0(0)			0(0)	
Laboratory	26.8(11)			4.2(1)	
Both	73.1(30)			95.8(23)	
CVD (%) (n)	13(6)	8.7(4)		3.7(1)	7.4(2)
CVD Risk factors					
BMI (mean±SD)	25.8±4.6(42)	26.9±3.5(46)		28.6±7.8(25)	27.9±5.1(27)
Smoking (%) (n)					
Current	30.4(14)	15.2(7)		14.8(4)	7.4(2)
Ex-smokers	21.7(10)	23.9(11)		14.8(4)	25.9(7)
AH (%) (n)	60.9(28)	69.6(32)		59.3(16)	63(17)
SBP (mean±SD)	126.6±17.6(45)	139±18(46)		129±22(27)	133±22(27)
DBP (mean±SD)	71.1±11.1(45)	79±11(46)		74.2±9.3(27)	79±11(27)
PMean (mean±SD)	91.6±12.7(45)	91.8±12.5(46)		95.4±13.9(26)	90.4±11.6(27)
HR	71.6±12.7(45)	70±12(46)		72.6±9.8(27)	68±10(27)
Dyslipidemia (%) (n)	54.3(25)	63(29)		22.2(6)	22.2(6)
TCHOL (mean±SD)	203.3±60(44)	216±41(44)		154±40.8(23)	203±34(25)
LDL (mean±SD)	129±45.4(41)	130±35(43)		92.8±26.7(19)	119±32(25)
HDL (mean±SD)	52.8±17.6(42)	58±15(42)		47.7±17.9(19)	62±18(25)
TG (mean±SD)	135.2±62.7(42)	133±89(44)		118±45.4(23)	107±45(25)
Diabetes (%) (n)	13(6)	15.2(7)		3.7(1)	7.4(2)
Glu (mean±SD)	98.2±23.2(45)	99±20(43)		101.2±31.2(27)	97±14(23)
Laboratory features					
ESR (>20 mm/h) (%) (n)	78.3(36/46)	33.3(6/18)		77.8(21/27)	15.4(2/13)
ESR (mean±SD)	56.9±36(46)	19±13(18)		47.1±30.5(27)	15.9±6.8(13)
CRP (>5 mg/L) (n)	71.1(32/45)	7.7(1/13)		77.8(21/27)	10(1/10)
CRP (mean±SD)	40.3±57.8(45)	2.8±1.8(13)		44.3±57.5(27)	2.1±1.4(10)
Comorbidities related treatment (%) (n)					
BP lowering drugs					
Lipid modifying	56.5(26)	50(23)		51.9(14)	55.6(15)
Glucose lowering drugs	43.5(20)	37(17)		14.8(4)	14.8(4)
Disease related treatment (%) (n)	13(6)	15.2(7)		0(0)	7.4(2)
Glycocorticosteroids	80.4(37)	0(0)		63(17)	0(0)
Immunosuppressives	28.3(13)	0(0)		63(17)	0(0)

Table 4.3 *Baseline - at study entry - characteristics of patients per disease group as compared to their matched NI controls.*

	LVV & MVV (n=43)	*RA Controls (n=43)*		*SVV (n=23)*	*RA Controls (n=23)*
Age (mean±SD, years)	66±13	65±12		56±16	55±17
Gender (female %) (n)	86.1(37)	86.1(37)		52.2(12)	52.2(12)
Disease type (%) (n)		-	GPA	39.1(9)	-
GCA	76.7(33)		MPA	30.4(7)	
TAK	11.6(5)		EGPA	4.4(1)	
IgG4 aortitis	2.3(1)		HSP	8.7(2)	
PAN	9.3(4)		SKIN	13(3)	
			CV	4.4(1)	
Active disease (%) (n)	88.4(38)	88.4(38)		87(20)	87(20)
CVD (%) (n)	11.6(5)	9.3(4)		4.4(1)	13(3)
CVD Risk factors					
BMI (mean±SD)	26±4.7(39)	28.1±5.4(43)		29.4±8(23)	27.1±4.6(23)
Smoking (%) (n)					
Current	32.6(14)	32.6(14)		17.4(4)	17.4(4)
Ex-smokers	20.9(9)	20.9(9)		13(3)	39.1(9)
AH (%) (n)	60.5(26)	55.8(24)		56.5(13)	60.9(14)
SBP (mean±SD)	127.1±17.8(42)	135±24(42)		127.8±22.2(23)	130±12(23)
DBP (mean±SD)	71.5±11.3(42)	77±16(42)		73.8±9.3(23)	79±8(23)
PMean (mean±SD)	92.4±12.8(42)	96.7±13.8(41)		93.9±13.8(22)	96.3±10.4(21)
HR (mean±SD)	72.7±12.5(42)	74±13(41)		72.7±8.9(23)	71±9(22)
Dyslipidemia (%) (n)	53.5(23)	46.5(20)		21.7(5)	17.4(4)
TCHOL (mean±SD)	207.6±59.8(41)	208±37(39)		158.8±42(19)	197±35(21)
LDL (mean±SD)	131.8±45.8(38)	132±33(37)		95.3±27(17)	113±30(18)
HDL (mean±SD)	53.6±17.6(39)	59±15(36)		45.8±16.8(17)	59±18(18)
TG (mean±SD)	139±63.4(39)	110±45(37)		123.8±41.4(19)	113±43(19)
Diabetes (%) (n)	11.6(5)	7(3)		4.4(1)	13(3)
Glu (mean±SD)	96.1±17.9(42)	90±11(39)		104.2±32.8(23)	97±17(23)
Laboratory features					
ESR (>20 mm/h) (%) (n)	79.1(34)	86.1(37)		78.3(18)	73.9(17)
ESR (mean±SD)	56.7±35.7(43)	37±20(43)		48.7±32.1(23)	26±13(23)
CRP (>5 mg/L) (n)	69.1(29/42)	52.5(21/40)		73.9(17)	59.1(13/22)
CRP (mean±SD)	37.9±56.1(42)	11.6±12.6(40)		49.3±61(23)	7.7±6.8(22)
Comorbidities related treatment (%) (n)					
BP lowering drugs	58.1(25)	46.5(20)		47.8(11)	47.8(11)
Lipid modifying	44.2(19)	30.2(13)		17.4(4)	27.3(3)
Glucose lowering drugs	11.6(5)	4.7(2)		0(23)	4.4(1)
Disease related treatment (%) (n)					
Glycocorticosteroids	79.1(34)	60.5(26)		60.9(14)	86.9(20)
Immunosuppressives	30.2(13)	74.4(32)		69.6(16)	78.3(18)

Table 4.4 *Baseline - at study entry - characteristics of patients per disease group as compared to their matched RA controls.*

	GCA (n=33)	*RA Controls (n=33)*	*GCA (n=24)*	*PMR Controls (n=24)*
Age (mean±SD, years)	71±9	70±8	70±8	70±8
Gender (female %) (n)	93.9(31)	93.9(31)	83.3(20)	83.3(20)
Active disease (%) (n)Clinical	90.9(30)	90.9(30)	91.7(22)	91.7(22)
CVD (%) (n)	15.2(5)	12.1(4)	4.2(1)	0(0)
CVD Risk factors				
BMI (mean±SD)	26.1±4.1(29)	27.3±4.3(33)	25.6±3.5(20)	27.3±4.5(22)
Smoking (%) (n)				
Current	33.3(11)	24.2(8)	29.2(7)	4.2(1)
Ex-smokers	21.2(7)	24.2(8)	33.3(8)	16.7(4)
AH (%) (n)	60.6(20)	57.6(19)	58.3(14)	66.7(16)
SBP (mean±SD)	129.2±16.8(33)	135±23(32)	130.3±17.3(24)	127.3±12.5(24)
DBP (mean±SD)	70.1±9.2(33)	76±17(32)	69.4±8.7(24)	70.6±7.8(24)
PMean (mean±SD)	91.5±12(33)	94.7±11.8(31)	90.9±13.1(24)	89.4±8.4(24)
HR (mean±SD)	72.7±13.5(33)	73±12(31)	70.9±10(24)	73±11(24)
Dyslipidemia (%) (n)	48.5(16)	51.5(17)	45.8(11)	45.8(11)
TCHOL (mean±SD)	208.4±56.3(32)	214±38(30)	210.3±59.9(23)	195.1±(20)
LDL (mean±SD)	131.1±45.4(31)	137±33(29)	130.5±46.4(23)	113.6±25.5(15)
HDL (mean±SD)	55.7±18.2(31)	58±15(28)	55±17.6(23)	67.2±12.4(15)
TG (mean±SD)	131.6±49.8(30)	113±48(29)	120.5±50.7(22)	111.3±38.6(18)
Diabetes (%) (n)	12.1(4)	9.1(3)	16.7(4)	8.3(2)
Glu (mean±SD)	95.4±17.7(33)	90,1±11.7(30)	100.9±27.9(24)	94.3±12.5(19)
Laboratory features				
ESR (>20 mm/h) (%) (n)	84.9(28)	90.9(30)	87.5(21/24)	75(18/24)
ESR (mean±SD)	63.2±35(33)	40±19.9(30)	65±35.2(24)	39.9±27.6(23)
CRP (>5 mg/L) (n)	71.9(23/32)	46.7(14/30)	69.6(16/23)	62.5(15/24)
CRP (mean±SD)	44.9±62(32)	9.9±10.2(30)	46.8±59.1(23)	17.9±26.9(23)
Comorbidities related treatment (%) (n)				
BP lowering drugs	57.6(19)	51.5(17)	50(12)	66.7(16)
Lipid modifying	42.4(14)	33.3(11)	41.7(10)	41.7(10)
Glucose lowering drugs	12.1(4)	6.6(2)	16.7(4)	8.3(2)
Disease related treatment (%) (n)				
Glycocorticosteroids	75.8(25)	60.6(20)	75(18)	70.8(17)
Immunosuppressives	18.2(6)	78.8(26)	16.7(4)	20.8(5)

Table 4.5 *Baseline - at study entry - characteristics of GCA patients and their matched controls RA and PMR controls.*

	All PSV (n=42)	*NIC (n=42)*	*All PSV (n=40)*	*RA Controls (n=40)*
Age (mean±SD, years)	59.4±12.1	58.8±11.5	58.5±11.6	57±11
Gender (female %) (n)	71.4(30)	71.4(30)	70(28)	70(28)
Disease type (%) (n)				
LVV & MVV	57.1(24)	-	55(22)	-
GCA	38.1(16)		35(14)	
TAK	9.5(4)		10(4)	
IgG4 aortitis	2.4(1)		2.5(1)	
PAN	7.1(3)		7.5(3)	
SVV	42.9(18)		45(18)	
GPA	16.7(7)		17.5(7)	
MPA	14.3(6)		15(6)	
HSP	2.4(1)		2.5(1)	
SKIN	7.1(3)		7.5(3)	
CV	2.4(1)		2.5(1)	
Active disease (%) (n)	69.1(29)	-	67.5(27)	67.5(27)
CVD (%) (n)	2.4(1)	2.4(1)	0(0)	5(2)
CVD Risk factors				
BMI (mean±SD, kg/m²)	28.8±6.0(41)	29.2±5.3(42)	28.9±6.1(39)	27.8±4.3(39)
Smoking (%) (n)				
Current	23.8(10)	16.7(7)	22.5(9)	22.5(9)
Ex-smokers	21.4(9)	19.1(8)	22.5(9)	27.5(11)
AH (%) (n)	59.5(25)	59.5(25)	60(24)	47.5(19)
Dyslipidemia (%) (n)	52.4(22)	50(21)	50(20)	57.5(23)
Diabetes (%) (n)	7.1(3)	4.8(2)	7.5(3)	7.5(3)
Laboratory features				
ESR (>20 mm/h) (%) (n)	57.1(24/42)	0(0/27)	55(22/40)	37.5(15/40)
ESR (mean±SD, mm/h)	43.9±(41)	11.7±(27)	43.3±35.4(39)	23.8±(40)
CRP (>5 mg/L)	54.8(23/42)	0(0/19)	52.5(21/40)	42.5(17/40)
CRP (mean±SD, mg/L)	34.8±57.2(41)	2.9±1.2(19)	35.6±58.4(39)	14.2±34.4(40)
Comorbidities related treatment (%) (n)				
BP lowering drugs	45.2(19)	45.2(19)	45(18)	35(14)
Lipid modifying	40.5(17)	38.1(16)	37.5(15)	37.5(15)
Glucose lowering drugs	4.8(2)	4.8(2)	5(2)	7.5(3)
Disease related treatment (%) (n)				
Glycocorticosteroids	61.9(26)	0(0)	62.5(25)	37.5(15)
Immunosuppressives	59.5(25)	0(0)	62.5(25)	100(40)

Table 4.6 *Baseline - at study entry - characteristics of all patients that underwent retinal vessel caliber evaluation with active and inactive disease state as compared to their matched NIC and RA controls.*

	GCA (n=16)	*NIC (n=16)*	*GCA (n=14)*	*RA Controls (n=14)*	*GCA (n=16)*	*PMR (n=16)*
Age (mean±SD, years)	66.3±7.9	64.9±7.9	64.7±7.1	64±8	66.3±7.9	67.4±7.1
Gender (female %) (n)	93.8(15)	93.8(15)	92.9(13)	92.9(13)	93.8(15)	93.8(15)
Active disease (%) (n)	81.3(13)	0(0)	78.6(11)	78.6(11)	81.3(13)	81.3(13)
CVD (%) (n)	6.3(1)	6.3(1)	0(0)	7.1(1)	6.3(1)	0(0)
CVD Risk factors						
BMI (mean±SD, kg/m^2)	27.2±3.1(15)	27.6±3.1	27.5±3.2(13)	26.3±5.2(13)	27.2±3.1(15)	27.1±3.4(16)
Smoking (%) (n)						
Current	31.3(5)	31.3(5)	28.6(4)	35.7(5)	31.3(5)	12.5(2)
Ex-smokers	31.3(5)	25(4)	35.7(5)	21.4(3)	31.3(5)	18.8(3)
AH (%) (n)	50(8)	50(8)	50(7)	50(7)	50(8)	68.7(11)
Dyslipidemia (%) (n)	68.8(11)	62.5(10)	64.3(9)	64.3(9)	68.8(11)	37.5(6)
Diabetes (%) (n)	12.5(2)	6.3(1)	14.3(2)	14.3(2)	12.5(2)	6.3(1)
Laboratory features						
ESR (>20 mm/h) (%) (n)	75(12/16)	0(0/10)	71.4(10/14)	50(7/14)	75(12/16)	68.8(11/16)
ESR (mean±SD, mm/h)	63.4±38.5(15)	12.6±7.0(10)	64.7±41(13)	23.8±13.8(14)	63.4±38.5(15)	38.4±23.3(15)
CRP (>5 mg/L)	62.5(10)	0(0/9)	57.1(8)	50(7/14)	62.5(10/16)	62.5(10/16)
CRP (mean±SD, mg/L)	49.4±76.6(15)	3.1±1.4(9)	54.2±81.5(13)	9.3±11.7(14)	49.4±76.6(15)	14.7±20.9(15)
Comorbidities related treatment (%) (n)						
BP lowering drugs	31.3(5)	37.5(6)	28.6(4)	50(7)	31.3(5)	62.5(10)
Lipid modifying	43.8(7)	50(8)	35.7(5)	57.1(8)	43.8(7)	37.5(6)
Glucose lowering drugs	6.3(1)	6.3(1)	7.1(1)	14.3(2)	6.3(1)	6.3(1)
Disease related treatment (%) (n)						
Glycocorticosteroids	62.5(10)	0(0)	64.3(9)	35.7(5)	62.5(10)	75(12)
Immunosuppressives	31.3(5)	0(0)	35.7(5)	100(14)	31.3(5)	6.3(1)

Table 4.7 *Baseline - at study entry - characteristics of GCA patients that underwent retinal vessel caliber evaluation with active and inactive disease state as compared to their matched NIC, RA and PMR controls.*

4.5b Large vessel vasculitis is associated with increased arterial stiffness.

Baseline PWV was compared between 63 PSV patients and 63 matched NIC as well as 54 PSV and 54 RA-controls. The detailed results are presented in Table 4.8 In the overall population, PWV of PSV patients was higher by 0.7 m/sec (i.e., suggesting a clinically meaningful difference) as compared to NIC (p=0.21), and by 1.3 m/sec to RA-patients (p=0.003). This tendency was more pronounced (1.2 m/sec and 1.9 m/sec) to those with LVV/MVV-(p=0.08 and p=0.001 respectively); on the contrary in SVV no difference to NIC and RA-control groups was found.

	All PSV (n=73)	*NIC (n=73)*	*P*	*All PSV (n=66)*	*RA Controls (n=66)*	*P*
PWV (All)	10.5±2.7(63)	9.8±3.0(63)	0.21	10.5±2.8(54)	9.2±1.9(54)	**0.003**
PWV (Active)	10.6±2.7(56)	9.9±3.1(56)	0.30	10.6±2.8(47)	9.3±2.0(47)	**0.013**
PWV (Inactive)	10.1±3.3(7)	8.7±1.3(7)	0.44	9.9±3.1(7)	8.2±1.3(7)	0.14
	LVV & MVV (n=46)	***NIC (n=46)***	***P***	***LVV & MVV (n=43)***	***RA Controls (n=43)***	***P***
PWV (All)	11.4±2.7(42)	10.2±3.1(42)	**0.08**	11.3±2.8(36)	9.4±1.9(36)	**0.001**
PWV (Active)	11.4± 2.6(37)	10.4±3.3(37)	0.14	11.4±2.7(32)	9.5±1.9(32)	**0.003**
	SVV (n=27)	***NIC (n=27)***	***P***	***SVV (n=23)***	***RA Controls (n=23)***	***P***
PWV (All)	8.9±2.1(21)	8.9±2.7(21)	0.28	8.9±2.1(18)	8.8±2.0(18)	0.66
PWV (Active)	9.0±2.1(19)	9.1±2.8(19)	0.35	8.9±2.2(15)	9.0±2.1(15)	0.89

Table 4.8 *Pulse wave velocity (PWV) according to disease type and activity at baseline. Baseline, at study entry, PWV compared to their matched non inflammatory and rheumatoid arthritis controls presented for: all patients PSV independently and according to disease state (active-inactive); per disease group separately: irrespectively and in active disease status.*

Further, sensitivity analysis showed that active giant cell arteritis-(GCA) patients had higher PWV by 1.0 m/sec (trend by p=0.16), 2.2 m/sec (p=0.001) and 1.7 m/sec (p=0.02) compared to NIC, RA and PMR-controls respectively (Figure 4.2A and Table 4.12). Interestingly, PSV patients with chronic, inactive disease exhibited increased PWV compared to either NIC (by 1.4 m/sec, p=0.44) or RA-controls (by 1.7 m/sec, p=0.14).

4.5c Pressure wave reflection is reduced in PSV and predominantly is SVV.

Augmentation index and AIx75 at study entry was evaluated in 64 and 65 PSV patients respectively and compared at 1:1 with that of NIC, while 56 and 57 patients were compared with equal number of RA-controls (Table 4.9). The total cohort exhibited lower AIx in both active and inactive disease status compared to either NIC (all, p=0.03, p=0.05, p=0.31, respectively) or RA-controls (trend but with no statistical significance: p=0.13, p=0.16, 0.60, respectively). Similarly, AIx75 tended to be lower in PSV

irrespectively to disease state compared to both NIC and RA-controls (Table 4.9). Classifying patients according to PSV type we noticed that SVV patients had more pronouncedly decreased AIx independently of inflammatory state (p=0.04, p=0.09) and in active disease (p=0.11, p=0.07) as compared to NI and RA-controls. On the other hand, patients with LVV/MVV had mildly lower AIx and AIx75 irrespectively of disease status and matched control group. Interestingly, GCA patients had lower AIx and AIx75 compared to NIC and RA controls and higher in both all and those with active disease when compared to PMR patients (no statistical significance) (Figure 4.2B, 4.2C, and Table 4.12).

	All PSV (n=73)	***NIC (n=73)***	***P***	***All PSV (n=66)***	***RA Controls (n=66)***	***p***
AIx (All) (n)	29.4±14.6(64)	33.9±10.3(64)	**0.03**	29±24.7(56)	33.8±12.5(56)	0.13
AIx (Active) (n)	29.7±14.3(57)	34.1±9.9(57)	**0.05**	29.2±14.4(49)	33.9±11.7(49)	0.16
AIx (Inactive) (n)	27.3±18.4(7)	32.7±13.8(7)	0.31	27.3±18.4(7)	33.5±18.5(7)	0.60
AIx75 (All) (n)	26.9±14.6(65)	29.8±10.1(65)	0.15	27±15(57)	30.6±11.3(57)	0.30
AIx75 (Active) (n)	27.5±14.3(57)	29.9±9.8(57)	0.26	27.7±14.7(49)	30.8±10.6(49)	0.47
AIx75 (Inactive) (n)	22.9±17.4(8)	28.7±12.7(8)	0.34	22.9±17.4(8)	29.6±15.9	0.30
	LVV & MVV (n=46)	***NIC (n=46)***	***P***	**LVV & MVV (n=43)**	**RA Controls (n=43)**	***p***
AIx (All) (n)	33.3±13.4(41)	36.9±8.8(41)	0.15	33.2±13.7(37)	35.9±10.9(37)	0.52
AIx (Active, all)	32.9±13.1(37)	36.4±8.3(37)	0.18	32.8±13.4(33)	34.9±10.5(33)	0.67
AIx75 (All) (n)	30.8±14(42)	32.9±7.3(42)	0.39	31.1±14.4(38)	32.7±9.2(38)	0.79
AIx75 (Active, all)	30.8±14(37)	32.6±7.7(37)	0.53	31.2±14.5(33)	31.8±8.9(33)	0.89
	SVV (n=27)	***NIC (n=27)***	**P**	**SVV (n=23)**	**RA Controls (n=23)**	**P**
AIx (All) (n)	22.5±14.4(23)	28.7±11.9(23)	**0.04**	20.7±13.3(19)	29.8±14.7(19)	**0.09**
AIx (Active, all)	23.7±14.8(20)	29.8±11.4(20)	0.11	21.8±13.8(16)	31.9±14(16)	**0.07**
AIx75 (All) (n)	19.9±13.3(23)	24±11.9(23)	0.11	18.7±13(19)	26.5±14(19)	0.17
AIx75 (Active, all)	21.4±13(20)	24.9±11.3(20)	0.2	20.4±12.7(16)	28.6±13.5(16)	0.15

Table 4.9 *Augmentation index (AIx) according to disease type and activity at baseline. Baseline, at study entry, AIx compared to their matched non-inflammatory and rheumatoid arthritis controls presented for all PSV patients independently and according to disease state (active-inactive); per disease group separately: irrespectively and in active disease status.*

4.5d Large vessel vasculitis is characterized by accelerated atheromatosis at the time of disease diagnosis.

Formation of atherosclerotic plaques at study entry was studied in 73 PSV patients, 73 NI and 66 RA controls. A detailed description per disease state, type, and vascular bed is presented in Table 4.10 Increased global (carotid, femoral, both) atherosclerotic plaque formation was observed evaluating all (p=0.005, p=0.02, P=0.008) as well as those patients with active disease (p=0.01, p=0.01, p=0.02) compared to NI-controls but only both carotid/femoral when compared to RA controls (p=0.01 and p=0.005 respectively). On the contrary that was not observed in patients with chronic (inactive) disease independently to control group. Separation according to disease type revealed increased plaque formation in all vascular beds in LVV/MVV patients (p=0.007, p=0.03, p=0.004), and only both carotid/femoral (p=0.03) compared to NI and RA controls respectively. Among LVV patients with active disease carotid and both carotid/femoral location was observed more frequently (p=0.03 and p=0.016 respectively), compared to NIC and carotid/femoral compared to matched RA (p=0.02). On the contrary, SVV patients exhibit mildly elevated plaque formation as compared to their NI and RA controls examined either all, or separately those with active disease . Interestingly, active GCA was associated with increased carotid/femoral plaque formation compared to NIC, RA and PMR matched controls (p=0.03, p=0.02, p=0.02) (Figure 4.2D and Table 4.12).

	All PSV (n=73)	*NIC (n=73)*	*P*	*All PSV (n=66)*	*RA Controls (n=66)*	*P*
Plaques (%) (n)	75.3(55/73)	54.8(40/73)	**0.01**	73.9(48/65)	69.2(45/65)	0.56
Carotid	61.6(45/73)	38.4(28/73)	**0.005**	61.5(40/65)	49.2(32/65)	0.16
Femoral	68.5(50/73)	49.3(36/73)	**0.02**	66.2(43/65)	52.3(34/65)	0.11
Both	54.8(40/73)	32.9(24/73)	**0.008**	53.9(35/65)	32.3(21/65	**0.01**
Plaques (Active)	75.4(49/65)	55.4(36/65)	**0.02**	3.7(42/57)	68.4(39/57)	0.54
Carotid	63.1(41/65)	40(26/65)	**0.01**	63.2(36/57)	45.6(26/57)	0.06
Femoral	67.7(44/65)	49.2(32/65)	**0.01**	64.9(37/57)	50.9(29/57)	0.13
Both	55.4(36/65)	33.9(22/65)	**0.02**	54.4(31/57)	(16/57)	**0.005**
Plaques (Inactive)	75(6/8)	50(4/8)	0.31	75(6/8)	75(6/8)	0.99
Carotid	50(4/8)	25(2/8)	0.31	50(4/8)	75(6/8)	0.31
Femoral	75(6/8)	50(4/8)	0.31	75(6/8)	62.5(5/8)	0.59
Both	50(4/8)	25(2/8)	0.31	50(6/8)	62.5(5/8)	0.62
	LVV &MVV (n=46)	***NIC (n=46)***	***P***	***LVV & MVV (n=43)***	***RA Controls (n=43)***	***P***
Plaques (All)	84.8(39/46)	65.2(30/46)	**0.03**	83.3(35/42)	78.6(33/42)	0.58
Carotid	73.9(34/46)	45.7(21/46)	**0.007**	73.8(31/42)	61.9(26/42)	0.25
Femoral	78.3(36/46)	45.7(21/46)	**0.03**	76.2(32/42)	59.5(25/42)	0.11
Both	67.4(31/46)	37(17/46)	**0.004**	66.7(28/42)	42.9(18/42)	**0.03**
Plaques (Active, all)	82.9(34/41)	65.9(27/41)	0.08	81.1(30/37)	75.7(28/37)	0.57
Carotid	73.2(30/41)	48.8(20/41)	**0.03**	73(27/37)	56.8(21/37)	0.15
Femoral	75.6(31/41)	56.1(23/41)	0.07	73(27/37)	56.8(21/37)	0.15
Both	65.8(27/41)	39(16/41)	**0.016**	64.9(24/37)	37.8(14/37)	**0.02**
	SVV (n=27)	***NIC (n=27)***	***P***	***SVV (n=23)***	***RA Controls (n=23)***	***P***
Plaques (All)	59.3(16/27)	37(10/27)	0.11	56.5(13/23)	52.2(12/23)	0.77
Carotid	40.7(11/27)	25.9(7/27)	0.25	39.1(9/23)	26.1(6/23)	0.35
Femoral	51.9(14/27)	37(10/27)	0.28	47.8(11/23)	39.1(9/23)	0.55
Both	33.3(9/27)	25.9(7/27)	0.55	30.4(7/23)	13(3/23)	0.16
Plaques (Active, all)	62.5(15/24)	37.5(9/24)	0.09	60(12/20)	55(11/20)	0.75
Carotid	45.8(11/24)	25(6/24)	0.14	45(9/20)	25(5/20)	0.2
Femoral	54.2(13/24)	37.5(9/24)	0.25	50(10/20)	40(8/20)	0.53
Both	37.5(9/24)	25(6/24)	0.35	35(7/20)	10(2/20)	0.07

Table 4.10 *Presence of atheromatic plaques according to disease type and activity at baseline. Baseline, at study entry, AI compared: (i) their matched non inflammatory and rheumatoid arthritis controls presented for: all patients PSV independently and according to disease state (active-inactive); per disease group separately: irrespectively and in active disease status.*

4.5e Intima Media Thickness of PSV/PMR patients and their matched controls

Baseline IMT evaluation was in 72 PSV/PMR patients, 72 NIC and 65 RA controls. The results are shown in detail in Table 4.11. Overall, IMT at the LCCA of all patients independently of disease and control group tended to be increased in both active and inactive disease state. After, classifying patients per disease group only those patients with LVV/MVV exhibited increased LCCA IMT when evaluated independently to baseline disease state (p=0.04; NIC and p<0.001; RA controls) as well as in active disease (p=0.13 NIC, p=0.003; RA controls). In addition, active GCA patients had

increased LCCA IMT compared to NIC, PMR and RA controls (p=0.22, p=0.006, p=0.04, respectively). (Figure 4.2E and Table 4.12).

	All PSV (n=73)	***NIC (n=104)***	***P***	***All PSV (n=66)***	***RA Controls (n=66***	***P***
IMT LCCA (n)						
All	0.84±0.3(72)	0.77±0.2(72)	0.1	0.84±0.3(65)	0.70±0.2(65)	**0.001**
Active	0.83±0.3(64)	0.78±0.2(64)	0.24	0.83±0.3(57)	0.70±0.1(57)	**0.003**
Inactive	0.92±0.4(8)	0.69±0.2(8)	0.18	0.92±0.4(8)	0.69±0.2(8)	0.1
	LVV & MVV (n=46)	***NIC (n=46)***	***P***	***LVV & MVV (n=43)***	***RA Controls (n=43)***	***P***
IMT LCCA (n)						
All	0.92±0.3(45)	0.80±0.2(45)	**0.04**	0.92±0.4(42)	0.70±0.1(42)	**<0.001**
Active	0.90±0.3(40)	0.81±0.2(40)	0.13	0.89±0.4(37)	0.70±0.1(37)	**0.003**
	SVV (n=27)	***NIC (n=27)***	***P***	***SVV (n=23)***	***RA Controls (n=23)***	***P***
IMT LCCA (n)						
All	0.69±0.2(27)	0.71±0.2(27)	0.39	0.70±0.2(23)	0.69±0.2(23)	0.49
Active	0.70±0.2(24)	0.72±0.2(24)	0.48	0.70±0.1(20	0.70±0.2(20)	0.72

Table 4.11 *Intimal-medial-thickness in PSV/PMR patients and their matched controls at baseline. Baseline, at study entry, AI compared: (i) their matched non inflammatory and rheumatoid arthritis controls presented for: all patients PSV/PMR independently and according to disease state (active-inactive); per disease group separately: irrespectively and in active disease status.*

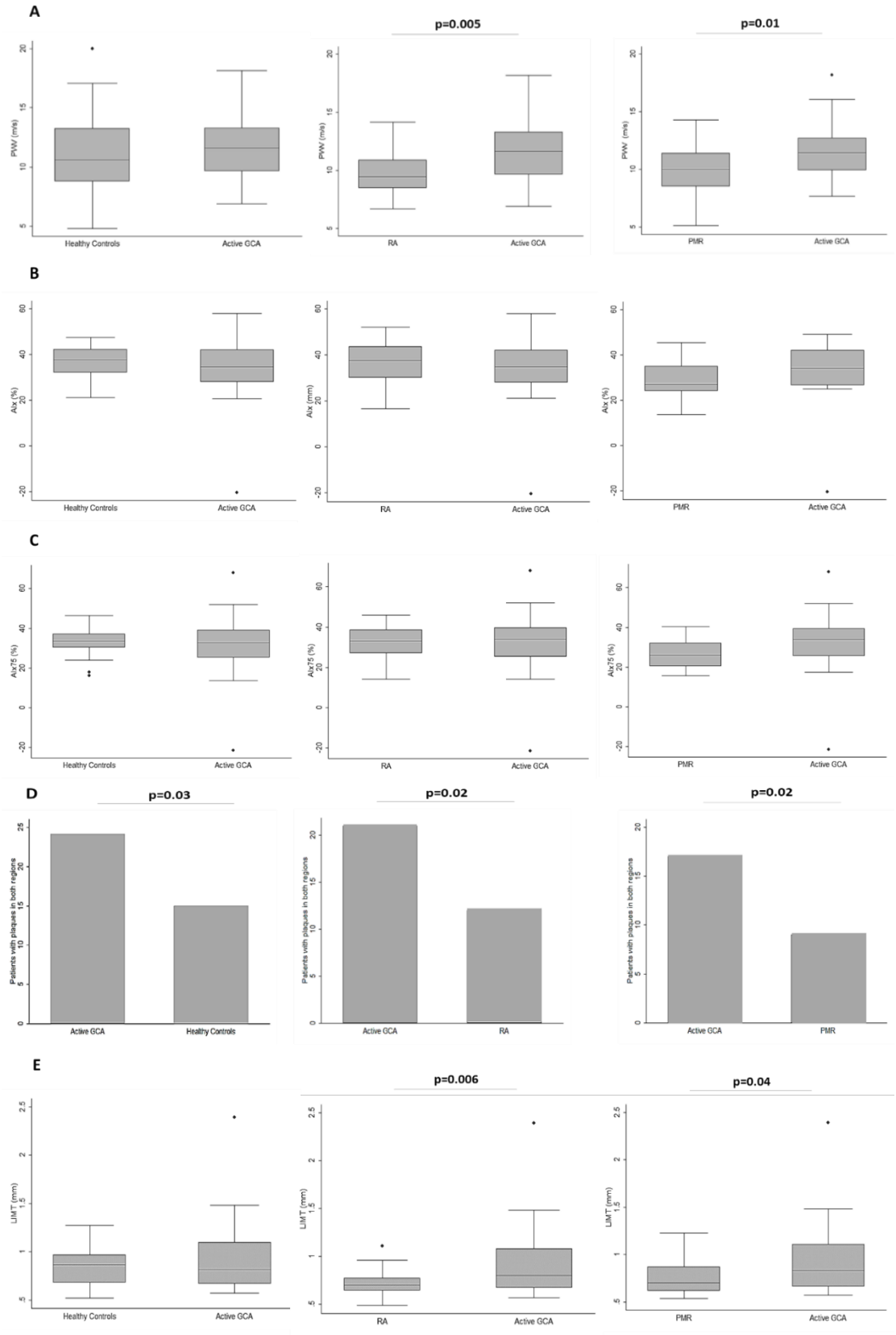

Figure 4.2 *Alterations of the macro-circulation in active GCA*
Comparison vascular biomarkers between active GCA and matched NIC, RA and PMR controls: 1A) PWV, 1B) AIx, 1C) AIx75 between active GCA and controls, 1D) plaques formation in both carotid and femoral beds, 1E) LCCA IM.

	GCA (n=36)	*NIC (n=36)*	*P*	*GCA (n=33)*	*RA Controls (n=33)*	*P*	*GCA (n=24)*	*PMR (n=24)*	*P*
PWV (All)	11.8±2.6(34)	10.8±3.2(34)	0.16	11.8±2.7(28)	9.6±1.8(28)	**0.001**	11.9±2.6(23)	10.2±2.6(23)	**0.02**
PWV (Active)	11.6±2.6(31)	10.9±3.3(31)	0.30	11.6±2.8(26)	9.7±1.8(26)	**0.005**	11.7±2.6(21)	9.9±2.4(21)	**0.02**
AIx (All) (n)	33.8±13.6(35)	37.3±6.8(35)	0.10	33.8±14(31)	36.5±9.9(31)	0.60	33.4±14.4(21)	30±9.4(21)	0.43
AIx (Active, all)	34±13.4(32)	36.9±6.9(32)	0.17	34±13.8(28)	35.9±9.6(28)	0.76	32.4±14.2(19)	28±8.4(19)	0.30
AIx75 (All) (n)	31.8±14.3(35)	33.3±6.0(35)	0.47	32.4±14.7(31)	32.8±7.8(31)	0.82	32.8±15.7(24)	27.6±8.2(24)	0.18
AIx75 (Active, all)	31.6±14.7(32)	33.2±6.2(32)	0.57	32.2±15.2(28)	32.8±7.6(28)	0.77	32.1±16.2(22)	26.1±7.7(22)	0.17
Plaques (All)	86.1(31/36)	69.4(25/36)	0.1	84.4(27/32)	84.4(27/32)	0.99	91.7(22/24)	91.7(22/24)	0.99
Carotid	77.8(28/36)	50(18/36)	**0.02**	78.1(25/32)	65.6(21/32)	0.27	79.2(19/24)	66.7(16/24)	0.33
Femoral	80.6(29/36)	61.1(22/36)	0.07	78.1(25/32)	65.6(21/32)	0.27	87.5(21/24)	66.7(16/24)	0.1
Both	72.2(26/36)	41.7(15/36)	**0.01**	71.9(23/32)	46.9(15/32)	**0.04**	75(18/24)	41.7(10/24)	**0.02**
Plaques (Active, all)	84.9(28/33)	69.7(23/33)	0.15	82.8(24/29)	82.8(24/29)	0.99	90.9(20/22)	90.9(20/22)	0.99
Carotid	78.8(26/33)	54.5(18/33)	**0.04**	79.3(23/29)	62.1(18/29)	0.15	81.8(18/22)	63.6(14/22)	0.18
Femoral	78.8(26/33)	60.6(20/33)	0.11	75.9(22/29)	62.1(18/29)	0.26	86.4(19/22)	68.2(15/22)	0.16
Both	72.7(24/33)	45.5(15/33)	**0.03**	72.4(21/29)	41.4(12/29)	**0.02**	77.3(17/22)	40.9(9/22)	**0.02**
IMT LCCA (n)									
All	0.93±0.4(35)	0.83±0.2(35)	0.20	0.92±0.4(32)	0.71±0.1(32)	**0.002**	0.98±0.4(23)	0.76±0.2(23)	**0.03**
Active	0.94±0.4(32)	0.84±0.2(32)	0.22	0.93±0.4(29)	0.72±0.1(29)	**0.006**	0.98±0.4(21)	0.76±0.2(21)	**0.04**
Eyes (n)	31	31		27	26		31	31	
CRAE (All)	175.6±22.3	166.1±16.0	**0.059**	176.6±23.3	167.5±14.3	0.091	175.6±22.3	179.7±22.0	0.460
CRVE (All)	214.1±31.5	202.6±16.7	**0.080**	212.5±31.9	205.0±19.7	0.311	214.1±31.5	213.8±17.1	0.966
AVR (All)	0.83±0.08	0.82±0.06	0.762	0.84±0.06	0.82±0.07	0.404	0.83±0.08	0.84±0.08	0.474
Eyes (n)	25	25		21	20		25	26	
CRAE (Active)	178.3±23.0	165.7±17.8	**0.035**	180.1±24.2	170.7±13.5	0.131	178.3±23.0	178.3±22.7	0.999
CRVE (Active)	219.6±32.0	200.8±17.4	**0.014**	218.6±33.0	210.5±16.5	0.327	219.6±32.0	213.1±16.6	0.378
AVR (Active)	0.82±0.08	0.83±0.06	0.720	0.83±0.07	0.81±0.08	0.516	0.82±0.08	0.84±0.09	0.458

Table 4.12 *Alterations of the macro- and micro- circulation of GCA patients compare to their matched NIC, RA and PMR controls at baseline.*

4.5f Alterations of vascular biomarkers (PWV, AIx/AIx75 and IMT) in two time points (active versus inactive disease) and correlation with acute phase reactants.

Potential changes of the studied vascular biomarkers between active and inactive disease were evaluated in 33 PSV patients. Independently to disease type the most sensitive to change following the reduction of the inflammatory bulk biomarker tended to be the IMT LCCA that was found to be decreased in all vasculitis subgroups (no statistical significance) (Table 4.13). In the overall group but also after sensitivity analysis classifying patients according to disease type (data not shown) we observed that PWV exhibited only mild modifications in all disease groups with no statistical significance. On the contrary, AIx75 was found to be decreased in LVV/MVV (p=0.09) but increased in SVV patients with disease in remission (p=0.12).

	Active PSV (Baseline, n=33)	*Inactive PSV (1st visit, n=33)*	*P value*			
PWV (mean±SD) (n)	10.4±2.2(28)	10.3±2.8(28)	0.80			
AIx (mean±SD) (n)	33.3±14.2(24)	32.9±12.1(24)	0.62			
AIx75 (mean±SD) (n)	31.1±15.1(24)	28.4±11(24)	0.70			
MEAN IMT LCCA (mean±SD) (n)	0.89±0.4(29)	0.80±0.3(29)	0.27			
	Active LVV & MVV (Baseline, n=23)	*Inactive LVV & MVV (1st visit, n=23)*	*P value*	*Active SVV (Baseline, n=10)*	*Inactive SVV (1st visit, n=10)*	*P value*
PWV (mean±SD) (n)	10.8±2.1(20)	10.4±2.4(20)	0.59	9.5±2.4(8)	10.3±4.0(8)	0.33
AIx (mean±SD) (n)	36.7±10.5(16)	34.1±9.7(16)	0.62	26.5±18.5(8)	30.5±16.4(8)	0.15
AIx75 (mean±SD) (n)	36.1±12.4(16)	29.3±8.7(16)	0.09	21.3±16.1(8)	26.8±15.3(8)	0.12
MEAN IMT LCCA (mean±SD) (n)	0.97±0.4(20)	0.88±0.3(20)	0.45	0.72±0.1(9)	0.64±0.1(9)	0.87

Table 4.13 *Alterations of vascular biomarkers in patients with active disease at baseline (study entry) and at the follow-up 1st visit with disease in remission (inactive disease) at baseline. Baseline, at study entry, PWV, AI/AI75 and IMT compared to themselves at 1st visit with disease in remission (active vs inactive disease state): all patients PSV/PMR independently and separately per disease group.*

Pearson's linear correlation coefficient analysis moving from active to inactive disease stage revealed bivariate correlation only between IMT-LCCA and CRP in SVV patients. The detailed results between the linear correlation of all the above non-invasive vascular biomarkers with the standard of care acute phase reactants (ESR and CRP) are presented in Table 4.14.

4.14A		*Active PSV (n=65)*	*Active LVV & MVV (n=41)*	*Active SVV (n=24)*
PWV	**ESR** (r/p)	0.04/0.77	-0.12/0.48	0.2/0.41
	CRP (r/p)	-0.25/0.07	-0.3/0.08	-0.25/0.31
AIx	**ESR** (r/p)	0.13/0.34	0.09/0.61	0.03/0.91
	CRP (r/p)	-0.07/0.63	0.05/0.75	-0.33/0.16
AIx75	**ESR** (r/p)	0.16/0.22	0.11/0.5	0.09/0.71
	CRP (r/p)	-0.07/0.61	0.01/0.96	-0.3/0.2
IMT LCCA				
	ESR (r/p)	0.16/0.20	0.15/0.35	-0.06/0.8
	CRP (r/p)	0.04/0.75	0.08/0.61	-0.05/0.81
Plaques (Y/N)	**ESR** (OR/P)	1.01/0.64	1.001/0.94	1.01/0.85
	CRP (OR/P)	0.99/0.34	0.99/0.66	0.99/0.37
Carotid	**ESR** (OR/P)	0.99/0.43	0.99/0.82	0.97/0.1
	CRP (OR/P)	0.99/0.68	1.001/0.88	0.99/0.25
Femoral	**ESR** (OR/P)	1.01/0.56	1.001/0.92	1.001/0.7
	CRP (OR/P)	0.99/0.74	0.99/0.94	0.99/0.76
Both	**ESR** (OR/P)	0.99/0.54	0.99/0.86	0.97/0.13
	CRP (OR/P)	0.99/0.89	1.003/0.67	0.99/0.51

4.14B		*PSV (n=33)*	*LVV & MVV (n=23)*	*SVV (n=10)*
DPWV	**DESR** (r/p)	0.29/0.22	0.12/0.68	0.4/0.44
	DCRP (r/p)	0.25/0.24	0.22/0.37	0.02/0.96
DAIx	**DESR** (r/p)	0.3/0.23	0.19/0.55	0.54/0.27
	DCRP (r/p)	0.29/0.2	0.26/0.35	0.18/0.73
DAIx75	**DESR** (r/p)	0.04/0.86	0.08/0.81	0.55/0.25
	DCRP (r/p)	-0.04/0.87	0.18/0.53	0.3/0.61
IMT				
LCCA	**DESR** (r/p)	0.16/0.5	0.17/0.59	0.41/0.36
	DCRP (r/p)	0.11/0.58	0.07/0.79	**0.91/0.005**

Table 4.14

4.14A *Pearson's linear correlation coefficient (r), or odds ratio (OR), and p values, between non-invasive vascular biomarkers with standard of care inflammatory biomarkers (CRP or ESR), in all participants with active disease at baseline (study entry) examination, stratified according to the underlying disease.*

4.14B *Pearson's linear correlation coefficient (r) and p values between on the one hand the difference from active to inactive disease status of non-invasive vascular biomarkers and on the other hand the corresponding difference of c-reactive protein (DCRP) or erythrocyte (DESR) sedimentation rate.*

4.5g Alterations of the retinal microcirculation in PSV patients.

Retinal microcirculation in PSV is altered in both active and inactive disease state.

Baseline retinal vessel calibers (CRAE & CRVE) were compared between patients and their matched controls and are presented in Table 4.15. In the overall population PSV patients had increased CRVE (p=0.003) and CRAE (p<0.001) as compared to NIC and RA controls (p=0.04, p=<0.001) independently to disease type and state. Interestingly after separating patients according to disease type, we found that LVV/MVV had increased both CRVE and CRAE independently to disease state (all: p=0.004, p=0.011 and p=0.005, p=0.017 respectively) compared to NIC but only CRAE when compared to RA controls (p=0.018, p=0.054). Further sensitivity analysis revealed that GCA patients with active disease had increased CRVE and CRAE compared to NIC (p=0.014, p=0.035 respectively) as well as when compared to RA and PMR patients but with no statistical significance (Figure 4.3A-4.3B, Table 4.12). On the contrary SVV patients

displayed statistically significant increased CRAE but not CRVE irrespectively to disease state and control group (Table 4.15). In patients with chronic disease remission CRAE and not CRVE was found to be increased compared to NIC (p=0.029) and RA-controls (p=0.008); no subpopulation analysis was feasible due to the limited number of patients per disease type.

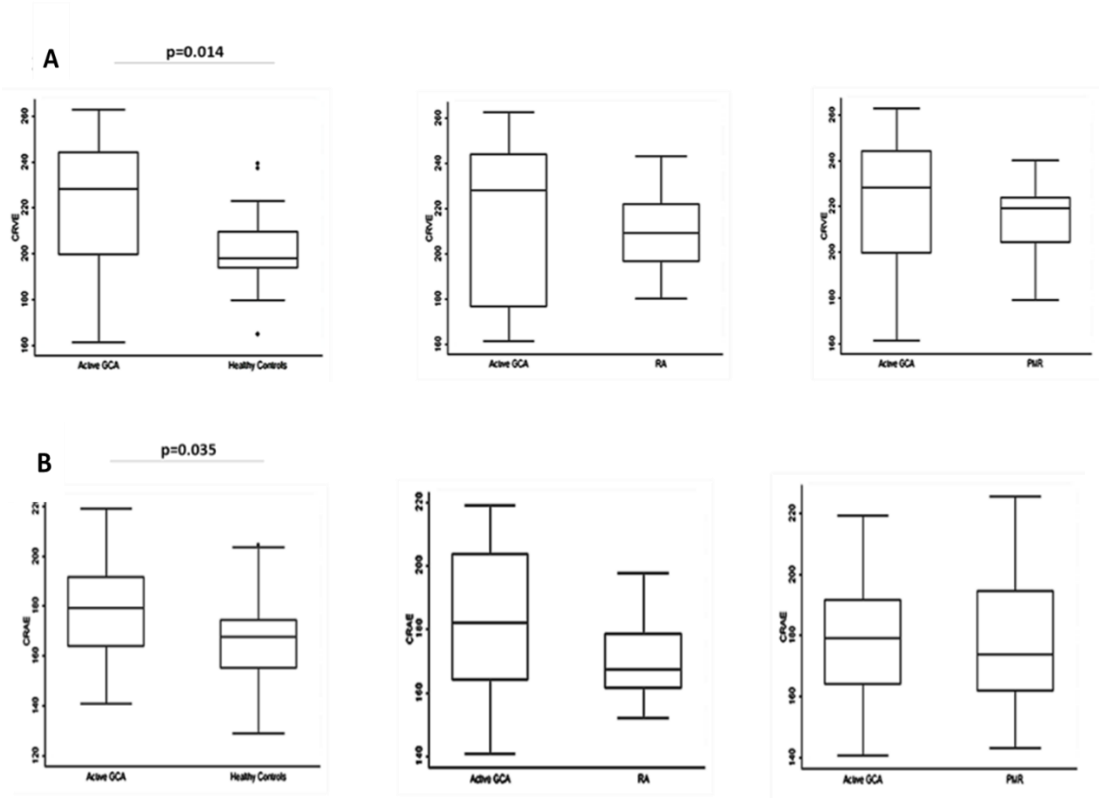

Figure 4.3 *Alterations of the retinal micro-circulation in active GCA.*
Comparison of CRVE (A) and CRAE (B) between active GCA and their matched NIC, RA and PMR controls.

	All PSV (n=42)	NI Controls (n=42)	P	Active PSV (n=29)	NI Controls (n=29)	P	Inactive PSV (n=13)	NI Controls (n=13)	P
Eyes (n)	82	81		57	56		25	25	
CRAE	179.8±17.4	166.2±15.3	**<0.001**	180.5±17.6	164.7±17.1	**<0.001**	178.3±17.1	169.4±9.8	**0.029**
CRVE	213.1±22.2	203.8±16.4	**0.003**	213.9±23.9	201.9±15.7	**0.002**	211.3±18.0	208.1±17.5	0.517
AVR	0.85±0.07	0.82±0.07	**0.007**	0.85±0.08	0.82±0.07	**0.029**	0.84±0.06	0.82±0.06	0.104
	ALL PSV (n=40)	**RA (n=40)**	**P**	**Active PSV (n=27)**	**Active RA (n=27)**	**P**	**Inactive PSV (n=13)**	**Inactive RA (n=13)**	**P**
Eyes (n)	78	74		53	51		25	23	
CRAE	180.4±17.4	167.1±18.1	**<0.001**	181.4±17.6	169.6±18.1	**0.001**	178.3±17.1	163.9±18.7	**0.008**
CRVE	212.5±21.8	205.5±20.6	**0.043**	213.1±23.6	206.1±20.7	0.113	211.3±18.0	204.1±20.9	0.202
AVR	0.85±0.06	0.82±0.07	**0.003**	0.86±0.07	0.83±0.07	**0.028**	0.84±0.06	0.81±0.07	**0.032**
	All LVV/MVV (n=24)	**NI Controls (n=24)**	**P**	**Active LVV/MVV (n=15)**	**NI Controls (n=15)**	**P**	**ALL LVV/MVV (n=22)**	**ALL RA (n=22)**	**P**
Eyes (n)	47	46		29	29		43	40	
CRAE	177.5±20.5	167.9±14.6	**0.011**	178.6±21.6	166.2±16.6	**0.017**	178.3±20.9	168.9±14.0	**0.018**
CRVE	216.2±26.6	202.5±16.9	**0.004**	219.4±29.9	200.8±16.1	**0.005**	215.4±26.6	207.6±17.7	0.119
AVR	0.83±0.07	0.83±0.06	0.687	0.82±0.08	0.83±0.06	0.643	0.83±0.06	0.82±0.07	0.291
	Active LVV/MVV (n=13)	**Active RA (n=13)**	**P**	**ALL SVV (n=13)**	**NI Controls (n=13)**	**P**	**Active SVV (n= 14)**	**NI Controls (n=14)**	**P**
Eyes (n)	25	24		35	35		28	27	
CRAE	180.2±22.4	169.9±12.8	**0.054**	183.0±11.5	163.9±16.1	**<0.001**	182.5±12.3	163.2±17.9	**<0.001**
CRVE	218.5±30.4	211.2±15.9	0.292	209.0±13.6	205.5±15.8	0.326	208.2±14.0	203.2±15.4	0.212
AVR	0.83±0.06	0.81±0.07	0.277	0.88±0.06	0.80±0.08	**<0.001**	0.88±0.06	0.81±0.09	**0.001**
	ALL SVV (n=18)	**RA (n=18)**	**P**	**Active SVV (n=14)**	**Active RA (n=14)**	**P**			
Eyes (n)	35	34		28	27				
CRAE	183.0±11.5	166.6±22.5	**<0.001**	182.5±12.3	169.4±22.0	**0.010**			
CRVE	209.0±13.6	203.0±23.6	0.202	208.2±14.0	201.6±23.6	0.217			
AVR	0.88±0.06	0.82±0.07	**0.001**	0.88±0.06	0.84±0.07	**0.031**			

Table 4.15 *Retinal Vessel Calibers alterations according to disease type and activity at baseline. Baseline, at study entry, retinal vessel calibers compared to their matched NIC and RA controls per disease type and state.*

Retinal microcirculation in PSV is altered in a reversible (venules) and irreversible way (arterioles).

The evolution of retinal vessel calibers in active and inactive disease state was evaluated in 13 PSV patients and is presented in Figure 4.4. The retreat of inflammation after immunosuppressive treatment for ≤ 6 months reduced CRVE and CRAE of patients with inflammatory disease (p=0.026 and 0.069 respectively) (Figure

4.4A & 4.4B). Of note, patients in remission had still higher values of both CRVE and CRAE as compared to their matched NIC controls (p=0.001 or 0.002) (Figure 4.4A & 4.4B). Pearson's linear correlation coefficient in active disease state (at baseline examination) revealed a bivariate correlation only between CRVE and standard of care acute phase reactants (both ESR and CRP) (Table 4.16). Following subgrouping of patients per disease type, both CRVE and CRAE correlated with ESR only in the LVV-MVV group (cc/p=0.644/0.017and 0.717/0.006 respectively) (Table 4.16A). Applying the same model of correlation in those patients with measurements in both active and inactive stage comparing the difference of the above parameters between the two time points we found bivariate correlation only between DCRVE with DESR(cc/p=0.628/0.021) (Table 4.16B).

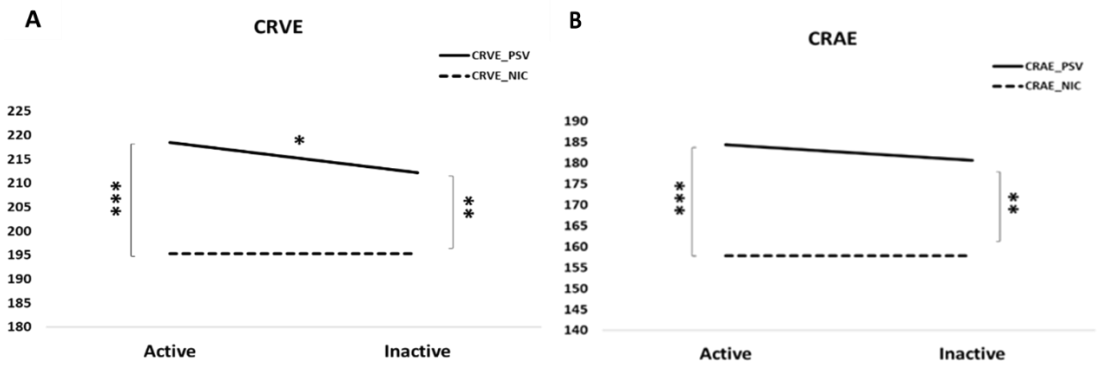

Figure 4.4 *Retinal vessel calibers in PSV patients with active disease at baseline (study entry) and available retina vessels' measurement at the time of disease remission (inactive disease).* ***A)*** *Alterations of CRVE (mean±SD) of 13 PSV patients (n=25 eyes) between active and inactive disease state (218±25.8 vs 212.2±21.2, p=0.026). PSV patients had increased CRVE when compared with NIC in both active (218±25.8 vs 195.3±15.1, p=<0.001) and inactive (212.2±21.2 vs 195.3±15.1, p=0.002) vasculitis status.* ***B)*** *Changes of CRAE (mean±SD, μM) of 13 PSV patients (n=25 eyes) between active and inactive disease state (184.4±19.7 vs 180.6±24.3, p=0.069). PSV patients had increased CRAE when compared with 13 NIC (n=26 eyes) in both active (184.4±19.7 vs 157.8±20.9, p=<0.001) and inactive (180.6±24.3 vs 157.8±20.9, p=0.001) vasculitis status. *=<0.05, **=<0.01, ***=<0.001.*

4.16A	**Active PSV (n=27)**	**Active LVV/MVV (n=13)**	**Active SVV (n=14)**	**Active GCA (n=11)**	**4.16B**	**PSV (n=13)**
CRAE					**DCRAE**	
ESR (cc/p)	0.322 / 0.101	**0.717/0.006**	-0.299/0.299	**0.692/0.018**	**DESR** (cc/p)	0.125/0.685
CRP (cc/p)	0.237 / 0.234	0.294/0.329	0.155/0.596	0.343/0.301	**DCRP** (cc/p)	-0.077/0.803
CRVE					**DCRVE**	
ESR (cc/p)	**0.541 / 0.004**	**0.644/0.017**	0.150/0.609	0.551/0.079	**DESR** (cc/p)	**0.628/0.021**
CRP (cc/p)	**0.528 / 0.005**	0.514/0.072	0.446/0.110	0.530/0.093	**DCRP** (cc/p)	0.519/0.069

Table 4.16 *Association of retina vessel calibers with ESR and CRP*
4.16A *Pearson's linear correlation coefficient (cc) and p values between retinal vessels calibers (CRAE or CRVE) and standard of care inflammatory biomarkers (CRP or ESR), in all participants with active disease at baseline (study entry) examination, stratified according to the underlying disease.* ***4.16B*** *Pearson's linear correlation coefficient (cc) and p values between on the one hand the difference from active to inactive disease status of central retinal arteriolar equivalent (DCRAE) or central retinal venule equivalent (DCRVE) and on the other hand the corresponding difference of c-reactive protein (DCRP) or erythrocyte (DESR) sedimentation rate.*

4.5h Discussion

This is the first study to explore comprehensively the functional and structural alterations of both micro- and macro- circulation, in PSV utilizing three state-of-the art, easily accessible, non-invasive techniques (ultrasonography, applanation tonometry and digital retinal images) in both active and inactive disease state, using as control groups individuals with no (NIC), mild (RA) and high inflammatory burden (PMR without underlying vasculitis). This approach allowed us to conclude that: i) aortic stiffness was statistically, clinically meaningful and irreversibly increased in LVV/MVV patients, ii) PWRs were altered in all and predominantly in SVV patients, iii) LVV/MVV patients presented increased burden of atheromatosis already upon disease diagnosis, iv) reversible upon remission common carotid remodeling (higher IMT) was observed in LVV/MVV, v) the retinal microcirculation exhibited vasodilation - in at least partly reversible (venules) and irreversible (arterioles) manner, associated with the level of the inflammatory response and irrespectively of the underlying disease-specific pathogenetic mechanisms, suggesting that digital retinoscopy might represent any easy direct tool to monitoring disease activity in PSV. Further sensitivity analysis revealed that GCA patients displayed increased aortic stiffness and accelerated

atheromatosis upon disease diagnosis as well as inappropriate carotid remodeling compared to either NIC, RA and PMR controls.

Aortic stiffening (AS), as assessed by carotid to femoral PWV, is an established maker of CVD mortality and morbidity in the general population (167), as well as, in several systemic autoimmune/autoinflammatory diseases (168). In the latter group AS is partially attributed to acute and chronic low-grade inflammation that induce functional and structural changes in the arterial wall (169). In PSV limited previous studies have shown evidence of increased PWV in TAK and BD patients, irrespectively to disease activity, while data are more controversial in the case of AAV (150) and scarce in GCA with only one study showing increased AS in inactive GCA with thoracic aorta dilatation (65). In line with these data, the present study displayed that LVV/MVV, but not SVV, patients have increased AS. Of note, the present evidence showed, that in contrast to other clinical disorders characterized by AS including among others RA, arterial hypertension (AH), and DM (170,171), in LVV/MVV patients AS is not reversible at least upon the early disease remission (first 6 months of this study follow-up duration). To this end, the hereby demonstrated increased AS in LVV/MVV by more than 1 m/sec, may suggest an overall increased CVD events risk by more than 7% (172). Moreover, patients with LVV/MVV should be regarded having long-term increased pulsatile stretch causing mechanical fatigue of the elastic components, thus rendering the aorta more prone to dilatation and aneurysm formation, a long-term complication seen in GCA even when the disease is quiescent (173). Therefore, more aggressive management of AH especially with “de-stiffening” drugs upon LVV diagnosis should be considered and addressed in future research.

PWRs, as assessed by AIx at the level of the aorta, are indirectly associated with aortic stiffness but also depend on heart rate and the peripheral circulation, since alterations in the reflection co-efficient at the level of small vessels and microcirculation moderate the generation of PWRs (174). Although PWV and AIx are thought to change in parallel, since PWV affects the timing of the merging incident and reflected waves, their association in PSV is not completely understood and often it is diverging (175). Booth AD et al, found that PWV and AIx were increased in active AAV and correlated with CRP (89), while Protogerou AD et al, showed that patients with active Behcet’s disease had decreased PWRs despite the increased aortic stiffness, due to peripheral

vasodilation (176), as also suggested previously by Vlachopoulos C et al. who demonstrated that acute inflammation increases arterial stiffness and decreases PWRs in healthy individuals (177). To our knowledge this is the first study investigating alterations in AIx in the whole spectrum of PSV. We identified different patterns of alterations in SVV compared to LVV/MVV. Patients with SVV, in the absence of any documented alterations in AS, presented lower AIx compared to NIC and RA controls. This finding was independent of heart rate, and moreover impaired AIx was at least partly restored upon disease remission. All together, these results could be attributed to peripheral vasodilatation, as further supported by our concurrent novel finding suggesting reversible dilatation of retinal microcirculation in SVV. On the other hand, in LVV/MVV patients minimal or no effect at all in AIx was observed, potentially because any reduction in PWRs imposed by peripheral vasodilatation of the microcirculation, as documented at the level of the retina, was counterbalanced by the increased PWV. These data are in line with those published by our group in BD previously (92), implying that low PWRs as measured by aortic AIx, might be a useful marker of monitoring PSV activity at the level of microcirculation.

Alterations of the retinal microcirculation have been independently associated with vascular risk factors and increased risk of CVD in the general population (150). Changes of the retinal vasculature and their association with disease activity have also been described in the context of systemic autoimmune rheumatic diseases with mild to moderate inflammatory activity (178,179). Previous studies in other than PSV diseases, have demonstrated an association between retinal venular widening and classic CVD risk factors including obesity, smoking, dyslipidemia, DM and atherosclerosis as well as chronic inflammation (180). Moreover, Moi JMY et al., provided evidence regarding the reversibility of this functional phenomenon after the subside of inflammation in RA patients (181), whereas Aissopou EK et al found that retinal microcirculation is spared in predominantly fibroproliferative vascular autoimmune disorders with low inflammatory component such as systemic sclerosis (182). After strict adjustment for the traditional CVD risk factors, our results confirmed the effect of inflammation on the retinal microcirculation for the first time in PSV, and showed a clear correlation between the vessel calibers, as well as the changes after treatment, and the inflammatory bulk. The overall present findings, comparing

patients with PSV, PMR, RA and NIC group, suggest that in the presence of systemic inflammation the retinal microcirculation (arterial and venous) exhibits - at least partly reversible - vasodilatation, which further support the hypothesis that these alterations are associated with the level of the inflammatory response, irrespectively of the underlying disease-specific pathogenetic mechanisms. Indeed, by applying strict matching to minimize vessel widening due to previously described confounding factors (180) we observed that the retinal microcirculation was affected at disease onset in PSV, independently of vasculitis type, suggesting that digital retinoscopy might represent any easy direct tool to monitor disease activity in PSV.

Inappropriate arterial wall remodeling leading to arterial hypertrophy, as measured by common carotid IMT may also contribute to local arterial stiffening in PSV patients and has been proposed by a single study, as an index of PSV activity, based on data derived from TAK (17,57). Limited data support the hypothesis of carotid thickening reversibility following treatment with steroids in GCA (63) or the contrary in AAV (88). The present study confirms previous findings, adding that IMT is increased in LVV/MVV and particularly in GCA, and mildly in SVV, compared to healthy NIC and RA controls. Moreover, in LVV/MVV patients IMT was significantly but not completely restored upon disease remission, demonstrating that tissue damage has an early reversible stage characterized by activation and accumulation of immune cells within the arterial wall, all producing a plethora of pro-inflammatory cytokines and thus amplifying the inflammatory cascade, as well as a late irreversible stage mediated by fibroblasts, smooth muscle and matrix alterations leading to arterial wall thickening and damage (153). The latter are also seen and further amplified in patients with deregulated AH (183), thus underlying the need for early suspicion and treatment of AH in LVV/MVV patients upon vasculitis diagnosis.

Moreover, the present findings confirm and further extend previous observations suggesting the presence of subclinical accelerated carotid/femoral atheromatosis in practically all types of PSV, even in SVV, although in the latter group the statistical significance was not reached due the small sample size. Most importantly our study demonstrated for the first time the presence of excessive carotid and femoral atherosclerotic plaques, especially in LVV/MVV patients, not only after long-term treatment, which has already been found and believed to be due to the chronic use of

steroids, but upon the very first diagnosis (active disease and naïve to treatment). One could claim that this is due to the increased age as well as the higher prevalence of CVD risk factors seen in these age groups. This concern is dually weakened in the present study as suggested by the careful age-matching with healthy NIC, PMR and RA controls as well as matching for all other confounding CVD risk factors, implying that the underlying vasculitic specific inflammatory process may induces accelerated plaque formation in these patients. Our finding raise even more clinical concerns since it suggests that long before the clinically overt symptoms, PSV may be silently accelerating the atheromatosis either due to long-term subclinical PSV or due to common genetical predisposition. The controversial benefit of early use of statins at the course of GCA must be further explored (184,185), however these findings suggest that early plaque detection must be incorporated in the CVD risk management of PSV.
The present study has some limitations. First, the inevitably small - due to the rarity of these diseases - sample size per disease group which could be a potent reason that some evaluations although exhibited a clear tendency, they did not reach statistical significance. Second, the short-interval between active disease and follow-up visit (disease in remission for a few months - 6 months at average) that may have not allowed evaluation of these biomarkers in long-term CVD prevention of PSV patients, however it must be said that most of these biomarkers respond to treatment within days, weeks, or a few months (171). Additionally, life and/or organ threatening complications at disease diagnosis imposed a short-term use of steroids and/or other immunosuppressive agents with unknown impact on micro- and macro-circulation. Finally, although all the above state-of-the art techniques allow a direct, cost-effective evaluation of circulation the degree of the examiner's subjectivity may be a concern.
Our study has multiple potential clinical implications that deserve to be addressed in the future. Each of the herein measured vascular biomarker (PWV, AIx, IMT and retinal vessel calibers) may have important role - in all or in specific types of PSV, as discussed above, including a) investigation of vascular damage pathways, b) treatment selection strategies, by monitoring disease activity at the vascular level beyond, clinical symptoms and blood biomarkers, and c) CVD risk stratification. In conclusion, the novel results of this study highlight the use of non-invasive biomarkers in early identification and management of CVD risk factors in PSV patients. Future studies with long term

follow-up and stratification according to immunosuppressive treatment will facilitate our better understanding of the link between vascular pathology and inflammation, yet providing new diagnostic, prevention and response treatment biomarkers.

4.5i Key messages

- Accelerated atheromatosis and arteriosclerosis may contribute to increased morbidity and mortality of PSV patients and is thought to be associated predominantly with long term of treatment with steroids.
- Non-invasive vascular biomarkers allow early identification of vascular pathology at disease diagnosis suggesting disease specific rather than treatment related CVD association.
- Early management of CVD risk factors as proposed by biomarkers assessing subclinical atheromatosis and arteriosclerosis could prevent long term CVD events in PSV patients.

CHAPTER 5. NMR-BASED METABOLOMICS IN GIANT CELL ARTERITIS AND POLYMYALGIA RHEUMATICA SEQUENCIAL SERA DIFFERENTIATES ACTIVE AND INACTIVE DISEASE

5.1 Objectives

Giant Cell Arteritis is the most prevalent form of systemic vasculitis in elderly. The activated immune system induces an acute systemic inflammatory response marked by elevated acute phase reactants (CRP and ESR), expansion and activation of various immune cell populations producing a variety of pro-inflammatory cytokines, growth and angiogenic factors. The combination of old age, along with the prolonged use of glucocorticosteroids and the, still not well understood, tissue remodeling, following the inflammatory response may create a variety of metabolic, vascular, and musculoskeletal comorbidities, affecting the quality and span of patients' lives (11,18,186,187).

Molecular medicine and application of system biology could give insights into the major unmet needs of the disease, including molecular stratification of patients towards precise targeted treatments, and identification of clinically relevant biomarkers, concerning both the inflammatory process and comorbidities. Metabolomics, the comprehensive evaluation of the metabolites in biological fluids and tissues and the unique for each individual metabolic "fingerprint", has emerged as a valuable tool to investigate the underlying mechanisms of complex diseases, as well as to monitor treatment efficacy and disease activity, with applications in several inflammatory diseases including inflammatory IBD (188), RA (189), fatty liver disease (190), and diabetes (191). Applications of metabolomics in vasculitis are still limited and have mainly focused on Kawasaki disease (192-194).

The current study aims to explore the metabolic profile of GCA patients' sequential sera and compare it to that of patients with increased inflammatory bulk such as those with PMR to uncover the operating metabolic pathways during the active and inactive disease state and to identify new candidate biomarkers. Finally, we aim to provide unique NMR based metabolic fingerprints of each disease following treatment with steroids.

5.2 *Patients' and Methods*

5.2a Study design

Serum samples of 33 GCA and 17 PMR patients, at 3-time points, i.e., time of diagnosis (active disease, V1), 1 month (V2) and 6 months (V3) of treatment with GCs (disease in remission), were collected. The number of serum samples included in visits V2 and V3 was reduced due to patients' personal inconvenience to meet the respective scheduled time point. In total, 110 serum samples from 50 patients were evaluated. GCA serum samples consisted of 33 treatment-naïve (25 females, mean age 73.0 ± 7.6 years and 8 males, mean age 69.5 ± 4.9 years), 22 in V2 and 21 in V3. PMR serum samples consisted of 16 naïve, 10 in V2 and 8 in V3 (9 females, mean age 65.0 ± 5.3 years and 8 males, mean age 77.0 ± 6.6 years). All patients included in the study fulfilled the respective international classification criteria (156,195), were naïve of treatment at baseline (V1) and were treated only with steroids, with no changes in comorbidities-related therapy until follow-up (V3). A descriptive analysis of baseline characteristics of all active patients at study entry are presented in Table 5.1. All patients were newly diagnosed with no disease flare until the last follow-up (V3). Due to the lack of a common activity index, as active disease was defined the presence of both clinical symptoms and increased acute phase reactants (ESR> 20 mm/h and CRP> 5 mg/L). The opposite was applied to define disease in remission. For patients with PMR the exclusion of underlying vasculitis was based on negative temporal artery biopsy and negative 18FDG-PET/CT. The study was approved by Ethics Committee of School of Medicine, National and Kapodistrian University of Athens, Greece (protocol no: 1718016656). All participants gave written informed consent for the collection and use of the samples, whereas the general data protection regulations and the Helsinki Declaration were routinely followed.

	GCA (N=33)	*PMR (N=17)*
AGE (MEAN±SD, YEARS)	70.7±7.1	70.5±7.2
GENDER (FEMALE %) (N)	75.8(25)	52.9(9)
BMI (MEAN±SD, KG/M^2) (N)	24.8±2.7 (31)	25.4±3.8(16)
SMOKING (%) (N)		
CURRENT	18.2(6)	23.5(4)
EX-SMOKERS	30.3(10)	19.4(5)
AH (%) (N)	42.4(14)	47(8)
DYSLIPIDEMIA (%) (N)	39.4(13)	41.2(7)
TCHOL (MEAN±SD, MG/DL) (N)	187.1±59.8(29)	188.9±32.2(14)
LDL (MEAN±SD, MG/DL) (N)	116.6±48.6(27)	111.4±26.4(12)
HDL (MEAN±SD, MG/DL) (N)	51.7±19.4(27)	62.5±12.5(12)
TG (MEAN±SD, MG/DL) (N)	112.2±49.6(28)	108.8±27.3(14)
DIABETES (%) (N)	9.1(3)	11.8(2)
GLUCOSE (MEAN±SD, MG/DL) (N)	99.2±15.5(29)	97.2±17.6(13)
LABORATORY FEATURES		
ESR (>20 MM/H) (%) (N)	96.9(32)	100(17)
ESR (MEAN±SD, MM/H) (N)	67±28.8(31)	61.1±26(17)
CRP (>5 MG/L) (N)	93.9(31)	94.1(16)
CRP (MEAN±SD, MG/L) (N)	58.1±62.6(31)	34.8±33.5(17)
COMORBIDITIES RELATED TREATMENT (%) (N)		
BP LOWERING DRUGS	42.4(14)	47(8)
LIPID MODIFYING DRUGS	39.4(13)	41.2(7)
GLUCOSE LOWERING DRUGS	9.1(3)	11.8(2)

Table 5.1 *Baseline characteristics of all active GCA and PMR patients.*

5.2b Materials and Methods

Reagents

Deuterium oxide (D2O) was purchased from Deutero GmbH (Kastellaun, Germany). Trimethylsilyl propionate (TSP), sodium phosphate dibasic (Na2HPO4) and hydrochloric acid (HCl) 37% were purchased from Sigma-Aldrich-Merck (Darmstadt, Germany). Sodium azide (NaN3) was purchased from Acros Organics- Thermo Fisher Scientific (Waltham, Massachusetts, U.S.). All chemicals were of analytical grade and dissolved in ultrapure water.

Sample preparation

Three hundred and fifty µL of serum sample was aliquoted and diluted to 700 µL with phosphate buffer pH=7.4 (adjusted using HCl 1N), containing 0.074 M Na2HPO4, 0.8 mg/mL TSP as internal standard and 0.4 mg/mL NaN3 as conservative. Mixing was achieved with gentle pipetting. An aliquot of 600 µL was then transferred to a 5-mm-diameter NMR tube. For the blank solution 350 uL of the phosphate buffer solution were mixed with 350 µL ultrapure water.

Metabolomics Data acquisition

17 For each sample, we performed one standard 1H 1D experiment with water suppression using the NOESY1D-pre-saturation pulse sequence with magnetic field gradients, as well as a 1H 1D T2-edited spectrum using the Carr-Purcell-Meiboom-Gill (CPMG) sequence that exploits the difference in the nuclear spin relaxation times between macromolecules and small molecules, resulting to reduced intensities for the broad signals of macromolecules and less distorted baselines. Moreover, we obtained its complementary 1H 1D T1-edited spectrum using the longitudinal encode-decode (LED) sequence, that reduces the intensities of the small molecules, resulting in a spectrum consisting mainly of the broad protein signals and thus used for the quantification of lipids. For the NMR experiments, 32 scans were acquired with a spectral width of SW = 12,019.23 Hz for CPMG and 18,028.85 Hz for LED and NOESY pulse sequences and a sampling of 98,304 data points, resulting in an acquisition time of 2.7 sec. A mixing time of 10 msec was used. Sample loading, temperature stability, field homogeneity, pulse calibration, data acquisition, and processing (including phase and baseline correction) were fully automated and controlled by the IconNMR v. 5.0.7 software (Bruker BioSpin GmbH, Rheinstetten, Germany). TopSpin 4.0.9 (Bruker BioSpin GmbH, Rheinstetten, Germany) was used for spectra visualization and chemical shift calibration to the anomeric 1H proton of α-glucose at 5.23 ppm from TSP. Total Correlation Spectroscopy (TOCSY) and Heteronuclear Single Quantum Coherence (HSQC) 2D NMR experiments were performed on the sample with the highest abundance of metabolites.

Metabolite Identification

The Chenomx NMR suite (Chenomx Inc., Alberta, Canada), a software that includes standard metabolite spectra, was utilized for the metabolite identification. 2D NMR experiments (TOCSY and HSQC), providing information on the spin systems and the 1H - 13C correlation respectively, also aided molecular identification and increased the confidence level of annotation. Signal assignment to each lipid class was based on previous literature (196) and work of our laboratory (197), as well as comparison with available spectral databases. For the quantification of small molecules the SMolESY-Select platform was also used (198). The statistical total correlation spectroscopy (STOCSY) analysis method, in MATLAB environment was utilized to trace glycoprotein signals in LED spectra.

Data Processing

A spectral bucketing of 0.01 at the range of δ = 8.80-0.40 was applied on the CPMG spectra using AMIX 4, (Bruker BioSpin GmbH, Rheinstetten, Germany). The region of water (δ 4.90-4.53) and peaks of contaminants, also present in the blank sample (δ 3.36-3.35, 2.73-2.71, 2.23-2.21, 2.08-2.06) have been removed, resulting in 797 buckets. For the LED spectra, a spectral bucketing of 0.001 at the range of δ = 5.60-0.30 was applied, to obtain optimal resolution. Afterwards, all spectra were normalized to total intensity.

Multivariate analysis

Principal Component Analysis (PCA), Partial Least Square-Discriminant Analysis (PLS-DA), and Orthogonal Partial Least Square-Discriminant Analysis (OPLS-DA) were applied in the normalized NMR data, using SIMCA-P 14.0 (Umetrics, Umea, Sweden). For the supervised methods (PLS-DA and OPLS-DA) the quality of the obtained models was assessed via R2X (variance explained by X Matrix) and Q2 (goodness of prediction) acquired by 7x cross-validation. In supervised methods, the validation of the generated models was checked through the performance of 100 random permutations and the corresponding quality parameters were compared to the corresponding of the original model. Data were either pareto-scaled or scaled to unit variance, whichever led to higher values of quality parameters. Selection of significant variables in the

multivariate analysis was based on VIP (Variable importance in projection) values. Only spectral variables with VIP > 1.5 were taken into consideration. Hierarchical clustering heatmap, as implemented in metaboanalyst.ca platform, was used to detect and perceive common metabolic features of the top 10 hits of lipid signals from mixed-effects modelling in the three different time points.

Univariate analysis

Statistical significance was tested for each identified metabolite from the SMolESY select platform (n=21 after exclusion of ethanol only present in some samples), as well as for the lipid signals from the LED spectra (n=20). Sample collection was not complete, and mixed-effects model approach, as implemented in Graphpad Prism 8, was utilized instead of repeated measures ANOVA on 30 GCA and 14 PMR patients. Fitting mixed-effects model allowed to include incomplete data sets (two measurements instead of three) because missing data were due to random reasons, not related to health issues. Outliers were excluded from analysis using the ROUT test. The Geisser-Greenhouse correction was used to adjust for lack of sphericity. Mann-Whitney test was applied for the comparisons of the two diseases in the same visit. The corresponding histograms were created using Graphpad Prism 8. A Bonferroni threshold was applied based on the number of tested variables ($p< 0.05/21= 0.0024$ and $p<0.05/20= 0.0025$ respectively).

Correlation coefficients with ESR and CRP

The correlation coefficient and the corresponding p-values of the 21 metabolites and 20 lipids with the ESR and CRP measurements, adjusted for intra individual variability, were calculated by applying repeated measures correlation (rmcorr) function in R environment. The common within-subject relationship was computed only for complete data sets with three measurements (13 GCA and 2 PMR patients for the ESR measurement and 12 GCA and 2 PMR patients for the CRP). A Bonferroni threshold was applied (metabolites: $p<0.0024$, lipids: $p<0.0025$). Receiver Operating Characteristic (ROC) Curves were plotted and the corresponding Area Under the Curve (AUC) were calculated to demonstrate the predictive performance of putative markers.

5.3 Results

Serum metabolomics of both GCA and PMR patients associate with disease activity

In total, 36 known metabolites including amino acids, organic acids, and carbohydrates, as well as 20 lipid and lipoprotein signals, were identified in the serum spectra. A distinct serum metabolic profiles were identified between active (V1) and inactive disease state (V2 and V3), independently of disease type (GCA or PMR). Multivariate approaches take advantage of the global nature of untargeted metabolomics, by incorporating all spectral information. The cross-validated scores plot of the Partial Least Square-Discriminant Analysis (PLS-DA) model revealed a clear discrimination, with good quality parameters and prediction power [R2X(cum)= 0.596, R2Y(cum)= 0.273, Q2= 0.219] (Figure 5.1A). Pairwise comparisons using orthogonal PLS-DA (OPLS-DA), that corrects for the variation in the metabolomics data, also exhibited a discrimination between time of active disease and each one of the two time points of remission [V1 vs. V2: R2Y(cum)= 0.444, Q2(cum)= 0.362; V1 vs. V3 treatment: R2Y(cum)= 0.537, Q2(cum)=0.445]. Detailed examination of the S-plot derived from the 6-month OPLS-DA model highlighted the metabolites with the highest discriminating power. N-acetylglycoproteins were detected in active disease and cholines in inactive disease, following steroid treatment (Figure 5.1B).

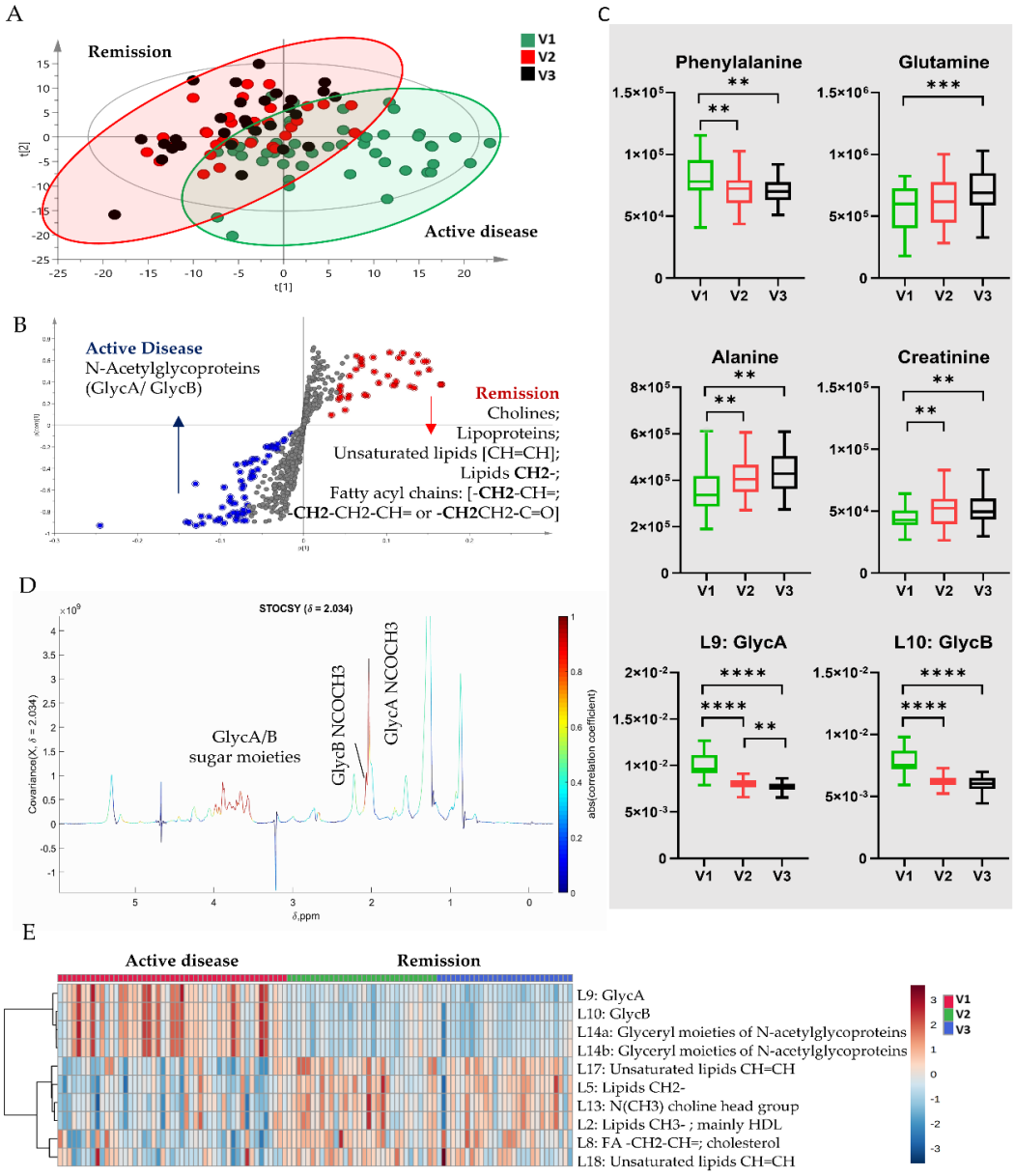

Figure 5.1 *Classification of GCA/PMR patients by disease activity/ visit. A) PLS-DA scores plot of serum CPMG NMR untargeted metabolomics data classified per visit, B) S-plot from the OPLS-DA analysis of V1 vs. V3 patients. Spectral variables on the up-right corner (red dots), assigned to cholines in bound phospholipids, lipoproteins (LDL, HDL) and signals from fatty acyl chains, were significantly increased in remission (V3). Spectral variables on the down-left corner (blue dots), assigned to N-acetylglycoproteins, were significantly increased in active disease (V1). C) Boxplots of significantly altered metabolites and lipids. Asterisks indicate statistical significance: **** p<0.0001, ***0.0001<p<0.001, **0.001<p<0.01, *0.01<p<0.05. D) STOCSY plot created from the analysis of LED spectra using δ 2.034 of GlycA as driver peak. E) Heat map created using the top 10 lipid signals from mixed-effects modelling. V1: Diagnosis (active disease, red); V2: After 1 month in steroid treatment (remission, green); V3: after 6 months in steroid treatment (remission, blue).*

We further quantified 21 metabolites in the NOESY spectra using the "SMolESY-select" 184 platform that enhances the resolution of small molecules. Due to incomplete data sets, fitting to mixed-effects model applied to 30 GCA and 14 PMR patients. The analysis unveiled increased phenylalanine (V1 vs. V2: p =0.0011) and decreased glutamine (V1 vs. V3: p=0.0009), alanine (V1 vs. V3: p =0.0020) and creatinine (V1 vs. V3: p=0.0016) after Bonferroni correction, in active disease (Figure 5.1C). The quantified metabolites and their inter-correlations are shown in Figure 5.2 while comprehensive histograms per disease of the significantly altered metabolites are shown in Figure 5.3.

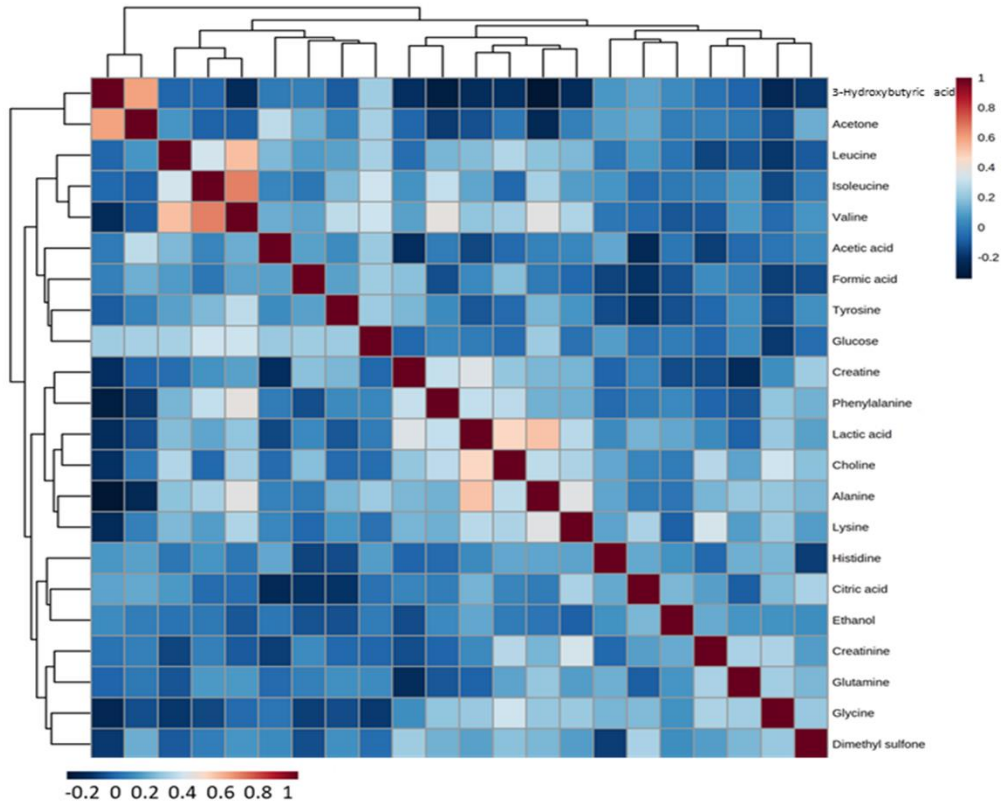

Figure 5.2 *Analysis of small molecules. Correlation between the metabolites quantified using the SMolESY-select. Ketone bodies (3-hydroxybutyrate and acetone) and branched chain amino acids (valine, leucine, isoleucine) are strongly correlated and clustered together.*

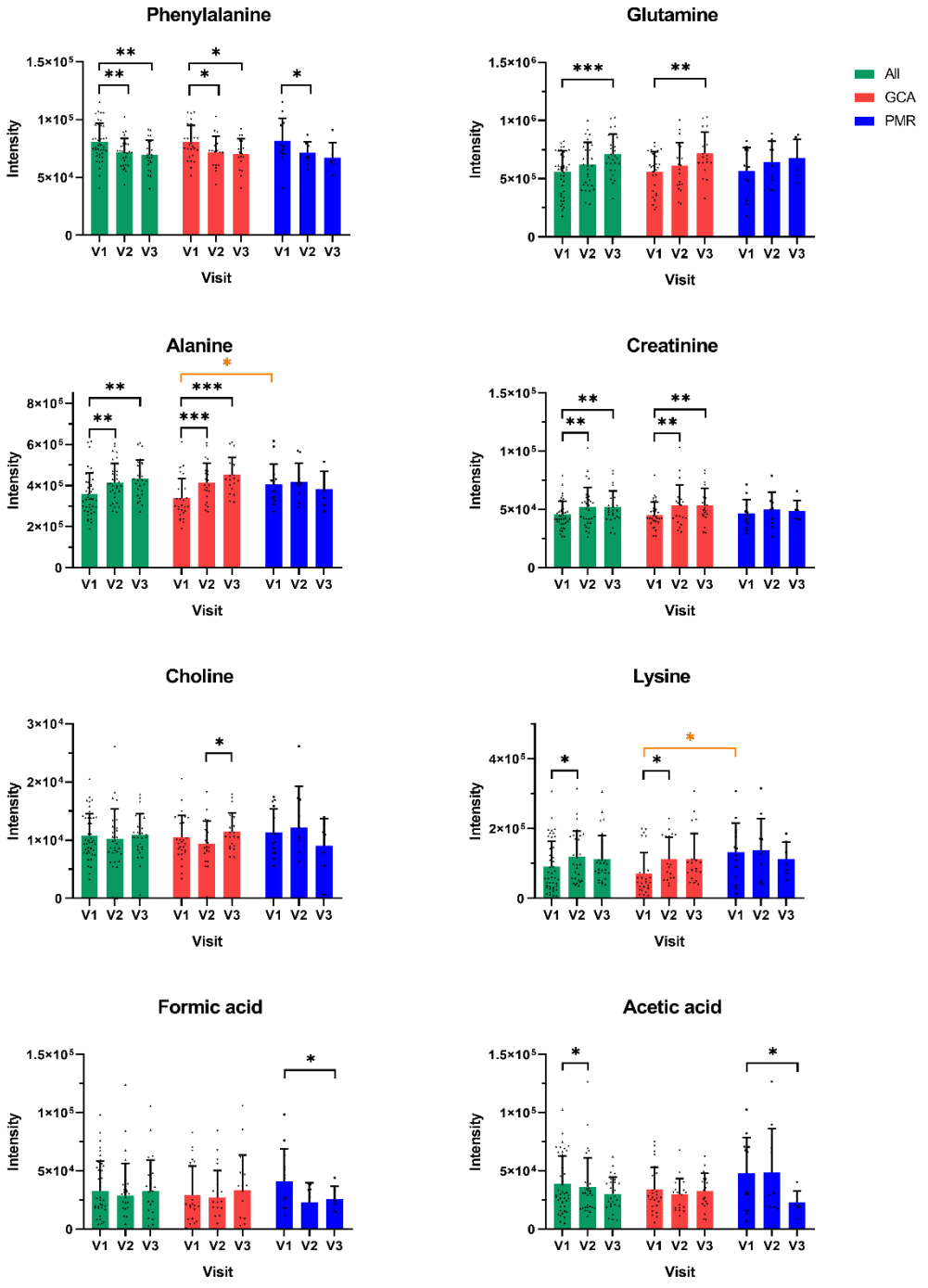

Figure 5.3 *Detailed histograms of significantly altered metabolites. V1: Diagnosis (active disease); V2: After 1 month in steroid treatment (remission); V3: After 6 months in steroid treatment (remission). Asterisks indicate statistical significance: ****p<0.0001, ***0.0001<p<0.001, **0.001<p<0.01, *0.01<p<0.05. Green: All samples; Red: GCA only; Blue: PMR only; In orange: GCA vs. PMR comparison in the same visit.*

Signals from the GlycA and GlycB N-acetyl glycoproteins were increased in active disease ($p<0.0001$) (Figure 5.1B,C). Instead, choline, derived from lipoprotein-bound serum phospholipids, was increased in remission ($p<0.0001$), in accordance with the multivariate analysis results (Figure 5.1B). STOCSY approach was applied on the driver peak of GlycA at δ 2.043 (Figure 5.1D) to identify resonances from the same structural unit and aid signal annotation in LED spectra. Other lipid signals, including lipoproteins, unsaturated lipids and CH2- signals of lipids were also significantly higher. However, these signals were poorly resolved and can only provide information on the chemical moieties present in several lipid classes and lipoproteins. To aid the visualization of the relation of glycoproteins' signals (L9, L10, L14a, L14b) with active disease, as well as lipoproteins' (L2) and certain lipids' (L5, L7, L8, L18) signals with remission periods, the relative concentrations of the top 10 hits from mixed-effects modelling are presented as a heat map in Figure 5.1E. Increased levels of all glycoproteins' signals (L9, L10, L14, L14a) were observed in active disease, while elevated lipid signals were characteristic of both remission periods. Comprehensive histograms per disease of the significantly perturbed lipid, lipoprotein and glycoprotein signals per disease are provided in Figure 5.4.

Serum metabolomics of GCA patients are differentiated from PMR in remission

Multivariate models discriminated serum metabolic profiles of GCA and PMR patients at the inactive disease states (time points V2 and V3, Figures 5.5A and 5.5B, respectively), but not at the active disease (V1). The quality parameters and prediction power at the three time points were the following: Diagnosis: R2Y (cum)=0.991 and Q2 (cum)=0.363, 1 month: R2Y (cum)=0.996 and Q2 (cum)=0.841, and 6 months: R2Y (cum)=0.997 and Q2 (cum)=0.788. Additionally, active disease (V1) and remission (V3) metabolomes were compared for GCA and PMR separately, using MVA. Increased 3-hydroxybutyrate and decreased lactate levels were observed after a 6-month treatment period in PMR patients, but not in GCA patients. Univariate analysis, in agreement with MVA results, highlighted alterations of FA and lipids related to treatment (Figure 5.5C). GCA differentiated from PMR due to higher lipids and cholines (L13) in V2 and unsaturated lipids both in V2 and V3 (Figure 5.4). Triglycerides (L15a) were found significantly higher in PMR at V2. Glycoproteins, in both diseases, were

greatly reduced upon treatment. In V2, GCA patients exhibited increased glycoproteins compared to PMR.

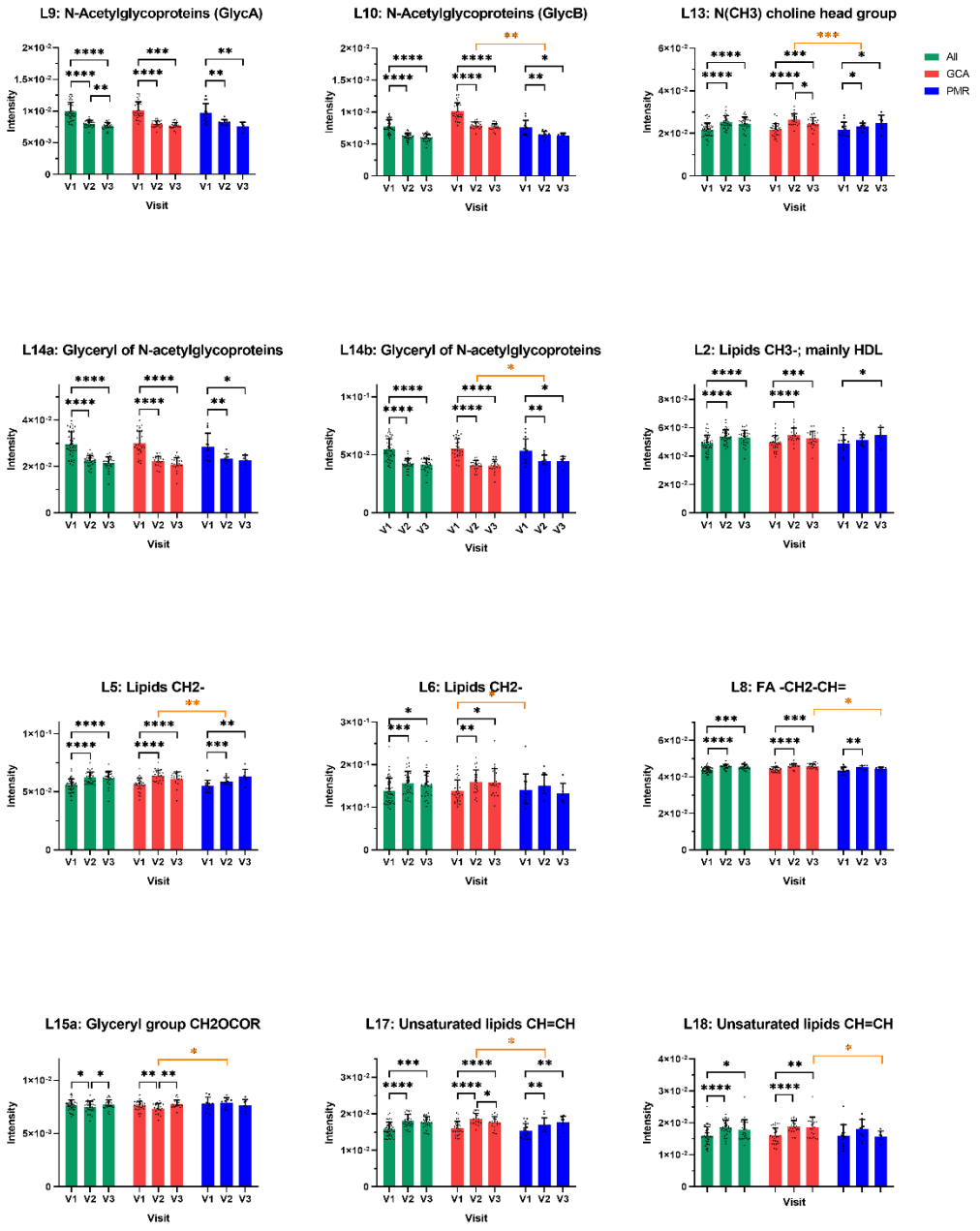

Figure 5.4 *Detailed histograms of significantly altered lipid signals. V1: Diagnosis (active disease); V2: After 1 month in steroid treatment (remission); V3: After 6 months in steroid treatment (remission). Asterisks indicate statistical significance: ****p<0.0001, ***0.0001<p<0.001, **0.001<p<0.01, *0.01<p<0.05. Green: All samples; Red: GCA only; Blue: PMR only; In orange: GCA vs. PMR comparison in the same visit.*

Besides lipids and FA, significant alterations as resulted from uni- and multivariate analyses were not consistent for other metabolites. More specifically, in UVA alanine and lysine were significantly higher in V1 for PMR patients compared to GCA (Figure 11C), although PMR and GCA metabolomes were not differentiated in V1 according to MVA. On the other hand, univariate analysis did not confirm the discriminant role of branched amino acids suggested by MVA. A higher number of participants and especially in the case of PMR, is required to reach statistically significant and consistent results.

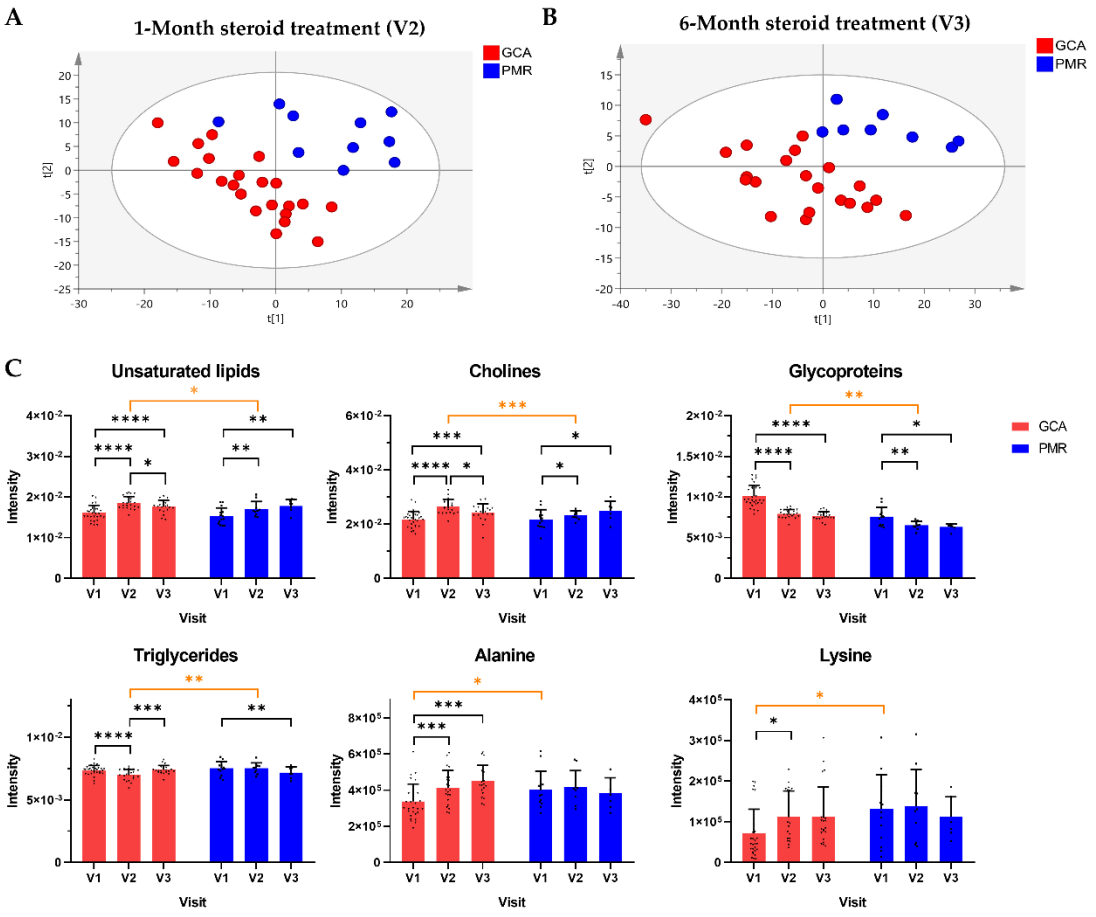

Figure 5.5 *Metabolic profiles of GCA vs PMR patients. A) PLS-DA scores plot of GCA vs. PMR patients at V2 after 1 month in steroid treatment (remission). B) PLS-DA scores plot of GCA vs. PMR patients at V3 after 6 months in steroid treatment (remission). C) Histograms of significantly altered lipids and metabolites between GCA and PMR patients according to Mann-Whitney test. Asterisks indicate statistical significance: **** p<0.0001, ***0.0001<p<0.001, **0.001<p<0.01, *0.01<p<0.05.*

Metabolic profiles correlate with markers of disease activity

Metabolites and lipid signals of 15 individuals with complete data sets, were subjected to repeated measures correlation analysis with CRP and ESR measurements. GlycA and GlycB exhibited the highest positive association with both ESR (r=0.91 with p-value= 1.68x10-12 and r=0.89 with p-value= 2.31x10-11, respectively) and CRP (r=0.75 with p-value= 3.08x10-06 and r=0.74 with p-value= 4.66x10-06, respectively), indicating the direct implication of these metabolites during acute inflammatory response. On the other hand, cholines in bound phospholipids, unsaturated lipids (CH=CH) and other lipid signals, exhibited a strong negative correlation with both ESR and CRP, suggesting a reverse association with disease activity. From the 21 tested metabolites, creatinine and alanine were negatively associated. Other metabolites associated with ESR and/or CRP ($p<0.05$) but did not reach the Bonferroni threshold, were glutamine, lysine, lactic acid, and creatine (negative) and 3-hydorxybutyric acid, phenylalanine, acetic acid and acetone (positive).

5.4 Discussion

This is the first study to investigate the metabolic profile in sequential sera of GCA and PMR patients in active and inactive disease phase, following treatment with GCs, exploiting NMR based metabolomics approaches. Metabolic fingerprinting revealed an important diversion before and after treatment (0, 1 and 6 months after treatment time points), as well as significant differences in specific serum metabolites. The identified metabolic patterns correlated with CRP and ESR. Although GCA and PMR could not be discriminated at the time of diagnosis, the two diseases can be discriminated based upon their metabolic profile, exhibiting different metabolic shifts, following the therapeutic intervention.

Our first task was to define markers of disease activity. Glycoproteins are involved in the acute phase response to an infection, sterile inflammation, or physical injury (199). Therefore, the levels of positive acute phase proteins and the complexity of the attached glycans increase

269 during inflammation (200). N-acetylglucosamine-(GlcNac) is a monosaccharide attached to serine or threonine residues of proteins by GlcNac transferase (OGT), while O-GlcNacase (OGA) can reversely remove it. This process is crucial in many inflammatory diseases, involving an array of inflammatory cells including macrophages, the proliferation and activation of T and B cells, neutrophils and natural killer cells (201). The acetyl moieties of GlcNacylated proteins display a characteristic NMR signal (GlycA) that has recently received much attention as a unique inflammatory biomarker displaying advantages over the existing clinical markers (202). It depicts an integrated signal of the GlcNac and N-acetylgalactosamine groups in the branches of five proteins: α-1-acid glycoprotein, α-1-antichymotrypsin, α-1-antitrypsin, haptoglobin, and transferrin. Several studies have proposed GlycA as a biomarker of systemic inflammation and its levels were elevated in several inflammatory conditions of various degree including rheumatoid arthritis (203), systemic lupus erythematosus (204), psoriasis (205), diabetes (206), and cardiovascular disease (207,208). Its composite nature and the low intra-individual variability may better reflect the heterogenous sources of chronic inflammation, compared to CRP (209,210). Our study suggests that in both the active phase of GCA and PMR, patients display high levels of GlycA that decrease following treatment, providing evidence that it can be used to monitor the inflammatory response.

During treatment with steroids an increase of lipoproteins was observed in remission points V2 and V3. Interestingly, the GlycA and GlycB signals displayed a strong negative correlation with the choline NMR signal derived from bound phospholipids. The latter is a composite signal attributed to supramolecular phospholipids (SPC) and specifically to phospholipid choline head groups -(N(CH3)3)+ of lysophosphatidylcholines bound to plasma glycoproteins and phosphocholines of specific High-Density Lipoprotein (HDL) subfractions. The ratio of GlycA to SPC has recently been reported as a sensitive biomarker for the presence of inflammatory response to SARS-CoV-2 infection (196). During the remission of inflammation following steroid treatment the decrease of acute phase glycoproteins was strongly correlated with the increase of SPC, probably due to increase of HDL subfractions rich in phosphocholines. HDL is reduced during inflammation, while HDL remodeling is amplified by secretory phospholipase A2,

produced during the acute phase, and hydrolyzes phosphatidylcholines to lysophosphatidylcholines modifying eventually the size distribution and function of lipoprotein particles (211,212).

It is interesting to note that the classical inflammation markers, ESR and CRP, besides the strong correlation with GlycA and GlycB, were found also associated with the ketone bodies 3-hyroxybutyrate and acetone. Ketone bodies are utilized as alternatives to carbohydrates energy sources when excessive energy demands are required. Ketone bodies were also shown to protect from oxidative stress in inflammatory conditions (213). Indeed, 3-hydroxybutyrate was found to exert its anti-inflammatory effects by blocking the NLRP3 inflammasome (214), in agreement with our correlation analysis results.

Regulatory role in inflammation is also known to possess free amino acids, through the activation of innate, adaptive, and regulatory immune responses (215). The hallmark of GCA is the presence of activated monocytes and T cells both in the periphery and the affected tissue. Accordingly, in the active state of the disease we observed decreased glutamine levels, a metabolite that is consumed mainly by proliferating cells such as the activated immune cells (216,217). Elevated phenylalanine in serum has also been observed in active inflammation and associates with increased mortality (218,219). A similar amino acid profile, with increased phenylalanine and decreased alanine and glutamine levels, has previously been described in Takayasu Arteritis, indicating common molecular pathways operating in the basket of large vessel vasculitides (220).

The PLS-DA analysis of GCA and PMR patients showed distinct metabolic profiles in remission, mostly attributed to different patterns of lipid metabolism. Lipids serve as an alternative source of energy and are implicated in cell signaling, modulating inflammatory responses, activation of immune cells and differentiation of macrophages (221,222). Several lipids are also involved in the synthesis of anti-inflammatory cytokines (223), while others may induce the synthesis of proinflammatory cytokines (224). However, one of the major issues accounting for these patients is the adverse effects of steroids (225,226). Therefore, the metabolic profile after steroid use is of equal importance with the active disease. The observed

alterations in lipid metabolism during steroid treatment may depict the initiation of steroid adverse effects including diabetes and hyperlipidemia, at the metabolite level, predicting future development of important comorbidities. Remarkably, the two groups exhibit characteristic differences in lipids during treatment, yet the different doses of corticosteroids administered to the GCA compared to the PMR patients may explain these differences.

Regarding GCA and PMR discrimination, it is interesting to note that phenylalanine appeared susceptible to treatment with glucocorticoids between the two disease groups. More precisely phenylalanine exhibits statistically significant difference between V1 -V2 and V1-V3 in GCA, while in PMR the only significant difference is observed between V1-V3. Phenylalanine has been related to inflammation, but to the best of our knowledge has not been previously linked to corticosteroids. This observation could be related to the disease and should be further investigated as a putative biomarker between GCA and PMR.

The main limitation of our study is the rather small number of patients per disease group and mainly for PMR. Moreover, since GCA and PMR patients were treated with different doses of corticosteroids, dose-dependent alterations of the metabolomic profile cannot be excluded. Our results, combined with future studies in both active and inactive disease, focusing mainly in the lipid profile, including NMR lipoprotein subclass analysis and mass spectrometry based lipidomics will highlight important molecules such as pro-resolving and anti-inflammatory lipid mediators (227). In addition, the study of patients receiving targeted treatments, as IL-6R inhibitor, might disclose different metabolites, relevant to active disease and therapy side effects, that can eventually be used as biomarkers. Finally, the integration of metabolomics to other up-stream high throughput approaches (proteomics, transcriptomics, epigenomics) (228), might give us further insights into stratifying with greater accuracy GCA and PMR patients.

CHAPTER 6. OCCURRENCE AND ANTIGENIC SPECIFICITY OF PERINUCLEAR ANTI-NEUTROPHIL CYTOPLASMIC ANTIBODIES (P-ANCA) IN SYSTEMIC AUTOIMMUNE DISEASES.

6.1 Objectives

Anti-neutrophil cytoplasmic antibodies are autoantibodies, mainly of IgG isotype, directed against proteins in the cytoplasmic granules of neutrophils and lysosomal proteins of monocytes. Depending on their staining pattern on alcohol-fixed neutrophils, ANCA are classified as diffuse cytoplasmic (C-ANCA), perinuclear (P-ANCA) and atypical (A-ANCA), the first two being highly significant for the diagnosis of ANCA-associated vasculitides. Myeloperoxidase represents the major autoantigen recognized by PANCA, followed by neutrophil elastase, lactoferrin, cathepsin G, BPI, catalase and lysozyme, among others (229). C-ANCA targeting PR3 has been associated with GPA, whereas P-ANCA targeting MPO is associated with MPA. Patients with vasculitis and P-ANCA targeting MPO are most likely suffering from MPA (55–65%), followed by EGPA (30–40%) and GPA (20–30%) (230). Emerging evidence suggests that ANCA specificity associates with disease activity and may affect the clinical phenotype, as well as response to treatment, risk of relapse and long-term prognosis. To this end, MPA patients with MPO-ANCAs are more likely to develop isolated crescentic glomerulonephritis (231,232), pulmonary fibrosis and peripheral neuropathy (233,234), while MPO+-GPA patients have more frequently limited disease, without severe organ involvement, less need for cyclophosphamide or rituximab therapy and fewer relapses than those with proteinase-3 (PR3)-ANCA (235,236). Interestingly, reappearance of MPO-ANCAs indicates relapse in more than 75% of patients (237). Beyond MPA, P-ANCA have been described in a variety of other SARDs, as well as chronic infections (238). Indeed, MPO-ANCAs have been reported in SLE (9.3%) (239), RA (4–18%) (240), SS (<3%) (241) and SScl (<2.4%) (242). Their presence has been associated with vasculitic patterns of glomerulonephritis and/or pulmonary involvement, while other P-ANCA-specific autoantigens, such as lactoferrin, neutrophil elastase, cathepsin and lysozyme, have also been described, although without known clinical significance (241,243,244). In this context, P-ANCA and their distinct targets

may have a potential role in distinguishing clinical phenotypes, disease prognosis and/or treatment monitoring. The aim of this study was to investigate the occurrence and the autoantigenic targets recognized by P-ANCA in various SARDs including systemic vasculitides.

6.2 Materials and Methods

Patients' Characteristics

The sera that have been examined for ANCA positivity by indirect immunofluorescence (IIF) in two highly experienced Greek diagnostic immunology laboratories (Department of Pathophysiology, School of Medicine, National and Kapodistrian University of Athens—a laboratory participating in the annual European Consensus Finding Study (ECFS) for Autoantibodies in Rheumatic Diseases in the context of EULAR and the Department of Immunology and Histocompatibility, Evangelismos General Hospital, Athens, Greece) during the past two years have been included in the study. From a total of 550 patients who were evaluated, 82 were found to be positive for the presence of P-ANCA by IIF and were included in the study. The medical records of all P-ANCA(+) patients were retrospectively analyzed and cumulative clinical, laboratory and autoantibody profile data were collected. Patients were classified into various systemic autoimmune diseases based on international classification criteria (161,162,243,245-253). Subgroup analysis to identify clinical associations with P-ANCA autoantibodies was performed in terms of P-ANCA titers, type of autoimmune disease and comparison with control patients whenever applicable. The study was approved by the Ethics Committee of School of Medicine, National and Kapodistrian University of Athens, Greece (protocol no: 1718016656), following the general data protection regulations (GDPR) of European Union and the Helsinki Declaration principles. All sera samples were stored at -20°C immediately after sampling and kept there until use.

Detection of P-ANCA Specificity and Associated Antigen Reactivity in Serum

The presence and titer of P-ANCAs were evaluated by standard IIF analysis on alcohol fixed neutrophils using the NOVA Lite ANCA kit, Inova Diagnostics Inc. (San Diego, CA, USA) according to the manufacturer's instructions, followed by evaluation of the

staining pattern by fluorescence microscopy. Positive sera at a dilution of 1:20 (positive cut-off threshold) were serially diluted until becoming negative and the last positive dilution was considered as the P-ANCA titer. The antigens recognized by P-ANCA were further evaluated by a commercially available multiplex ELISA (ANCA profile ELISA, Euroimmun, Lubeck, Germany), analyzing the reactivity against MPO, lactoferrin, neutrophil elastase, cathepsin G and BPI antigens, semi-quantitatively and according to the manufacturer's instructions.

Detection of ANA and Other Autoantibodies in Serum

Similar to ANCA detection, the presence and titer of ANA were evaluated by IIF using the NOVA Lite HEp-2 ANA kit, Inova Diagnostics Inc in serial serum dilutions starting from 1:160 dilution and fluorescence microscopy. Anti-Ro(SSA), anti-La(SSB), anti-Sm, anti-U1RNP, anti-Scl70 and anti-RibP(IgG) autoantibodies were tested by immunoblotting using the Euroline Anti-ENA ProfilePlus1 (IgG) and Euroline ANA Profile-3 kits, Euroimmun. The levels of IgG and IgM antibodies against cardiolipin (aCL), 2GPI and double-stranded DNA (ds-DNA) were determined by home-made ELISAs, as previously described (254-256). Anti-CCP, anti-TPO and anti-Tg were measured by commercially available ELISAs (QUANTA Lite CCP3.1 IgG/IgA, QUANTA Lite TPO and QUANTA Lite Thyroid T ELISA kits, Inova Diagnostics Inc.) according to the manufacturer's instructions, whereas RF was detected by agglutination assay using commercially available latex-based reagents (RapiTex RF, Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany) according to manufacturer's instructions.

Statistics

Comparisons of categorical data were performed by chi square or Fisher exact test when cell counts were <5. For continuous variables, the Shapiro–Wilk normality test was performed initially, followed by the Mann–Whitney–Wilcoxon or t-test accordingly. Statistical analyses were performed using Python 3.6, and R software 4.0.3.

6.3 Results

Patients' Characteristics

Eighty two of 550 tested patients were found to be positive for P-ANCA autoantibodies (in titer ≥1:20 dilution), 69 (84.2%) of whom fulfilled the criteria of a SARD and 13 (15.9%) who presented with HT. The 69 P-ANCA-positive SARD patients included 28 (40.6%) with SLE, 24 (34.8%) with a form of systemic vasculitis (18 with MPA, two each with BD and HSP, and one each with aortitis and CV, 7 (10.2%) with pSS , 5 (7.2%) with primary APS, 3 (4.4%) with RA, and one each with SSCL and sarcoidosis. The majority of SARD patients (57/69, 82.6%), as well as HT patients (11/13, 84.6%) were women. The median age at the time of P-ANCA measurement was 58 years (range: 20–85) for the SARD group and 55 years (range: 34–77) for those with HT patients.

P-ANCA Titers and Serum Autoantigen Specificity Per Autoimmune Disease

As detected by IIF, the titers of P-ANCA autoantibodies in the 82 P-ANCA-positive sera ranged from 1:20 to 1:640 (median: 640, Table 8). The majority of patients with SARD who were studied (50/69, 72.5%) presented with high P-ANCA-titer, namely, ≥1:80 (in 50/69, 72.5%) or ≥1:160 (in 43/69, 62.3%). Microscopic polyangiitis patients had higher P-ANCA titers compared to SLE patients (Figure 6.1). Among the 18 sera of PANCA-positive MPA patients, 11 (61.1%) presented reactivity to MPO (MPA-P-ANCAMPO-positive), whereas the remaining seven did not exhibit any reactivity against the various autoantigens examined (MPA-P-ANCA-NS). On the other hand, the vast majority of the patients with other SARD were not found to recognize any of the P-ANCA-related antigens under investigation, including 25/28 (89.3%) of SLE patients studied. In fact, monospecific P-ANCA-positive patient cases with anti-MPO reactivity included one each with SLE, APS, RA and systemic sclerosis, whereas one patient with SS reacted with elastase and a SLE patient with lactoferrin. In addition, a SLE patient had double specificity for MPO/lactoferrin (Table 6.2).

Patient Groups		P-ANCA Serum Titers (No Positive)					
		≥1:640	1:320	1:160	1:80	1:40	1:20
Vasculitides	MPA (n = 18)	9	5	1	1	1	1
	BD (n = 2)	1	1	0	0	0	0
	Aortitis (n = 1)	1	0	0	0	0	0
	HSP (n = 2)	0	0	1	1	0	0
	CV (n = 1)	0	1	0	0	0	0
SLE (n = 28)		9	6	3	7	23	0
APS (n = 5)		1	1	1	1	0	1
SS (n = 7)		5	0	0	0	0	2
RA (n = 3)		2	0	0	1	0	0
SSCL (n = 1)		0	1	0	0	0	0
Sarcoidosis	(n = 1)	0	0	1	0	0	0
Hashimoto	(n = 13)	2	1	4	2	1	3

Table 6.1 *Serum P-ANCA titers in the various P-ANCA positive autoimmune disease patients studied.*

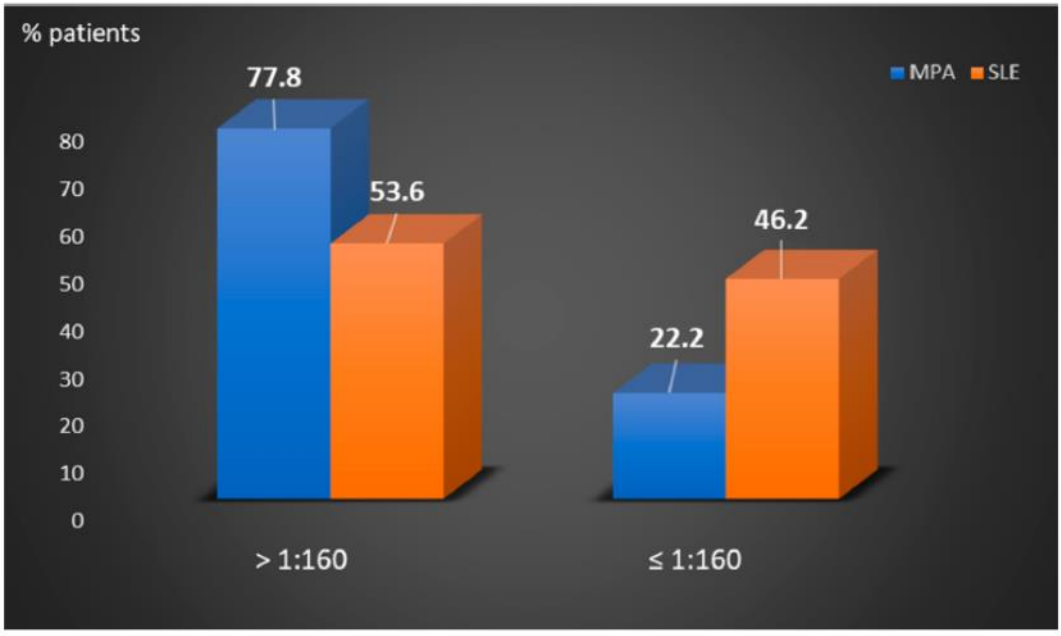

Figure 6.1 *Comparison of the persentages of P-ANCA positive MPA and SLE patients according to low ≤1/160 or high ≥1/160 titers.*

Autoimmune Diseases		Antigens Recognized by P-ANCA Positive Sera (No Positive)					
		MPO	Elastase	Cathepsin G	BPI	Lactoferrin	MPO/ Lactoferrin
Vasculitides	MPA (n = 18)	**11**	0	0	0	0	0
	BD (n = 2)	**1**	0	0	0	0	0
	Aortitis (n = 1)	0	0	0	0	0	0
	HSP (n = 2)	0	0	0	0	0	0
	CV (n = 1)	0	0	0	0	**1/1 (100)**	0
SLE (n = 28)		**1**	0	0	0	**1/28 (3.6)**	**1/28 (3.6)**
APS (n = 5)		**1**	0	0	0	0	0
SS (n = 7)		0	**1**	0	0	0	0
RA (n = 3)		**1**	0	0	0	0	0
SSCL (n = 2)		**1**	0	0	0	0	0
Sarcoidosis (n = 1)		0	0	0	0	0	0
Hashimoto (n = 13)		0	0	0	0	0	0

Table 6.2 *P-ANCA related antigenic specificities in the various P-ANCA positive autoimmune disease studied.*

Autoantibody Profile of P-ANCA Positive Patients and P-ANCA Related Specificity

Microscopic polyangiitis-P-ANCA patients had increased frequency of ANA with a titer ranging from 1:160 to 1:1280 [1:160: 30%, n = 3/10, 1:320: 50%, n = 5/10, 1:640: 10%, n = 1/10 and 1:1280: 10%, n = 1/10) and RF (27.8%, n = 5/18) (Figure 6.2a). Compared to MPA-P-ANCA-NS, MPA-P-ANCA-MPO-positive patients had higher prevalence of ANA (63.6%, n = 7/11 vs. 42.9%, n = 3/7) and RF (36.4%, n = 4/11 vs. 14.3%, n = 1/7) and lower prevalence of anti-2GPI-IgM (0% vs. 14.3%, n = 1/7), anti-Ro52/Ro60 (0% vs. 14.3%, n = 1/7) and anti-TPO (9,1%, n = 1/11 vs. 14.3%, n = 1/7), although these differences did not reach statistical significance (data not shown). The autoantibody profile of P-ANCApositive SLE patients is presented in Figure 6.2B. All were ANA positive (28/28), with the vast majority having anti-dsDNA antibodies (in 21/25, 75%) and anti-Ro60/SSA autoantibodies (in 14/28, 50%). The majority of P-ANCA-positive SS patients were ANA positive (5/7, 71.4%) and approximately half of them had also anti-Ro52/Ro60 autoantibodies (3/7, 42.9%).

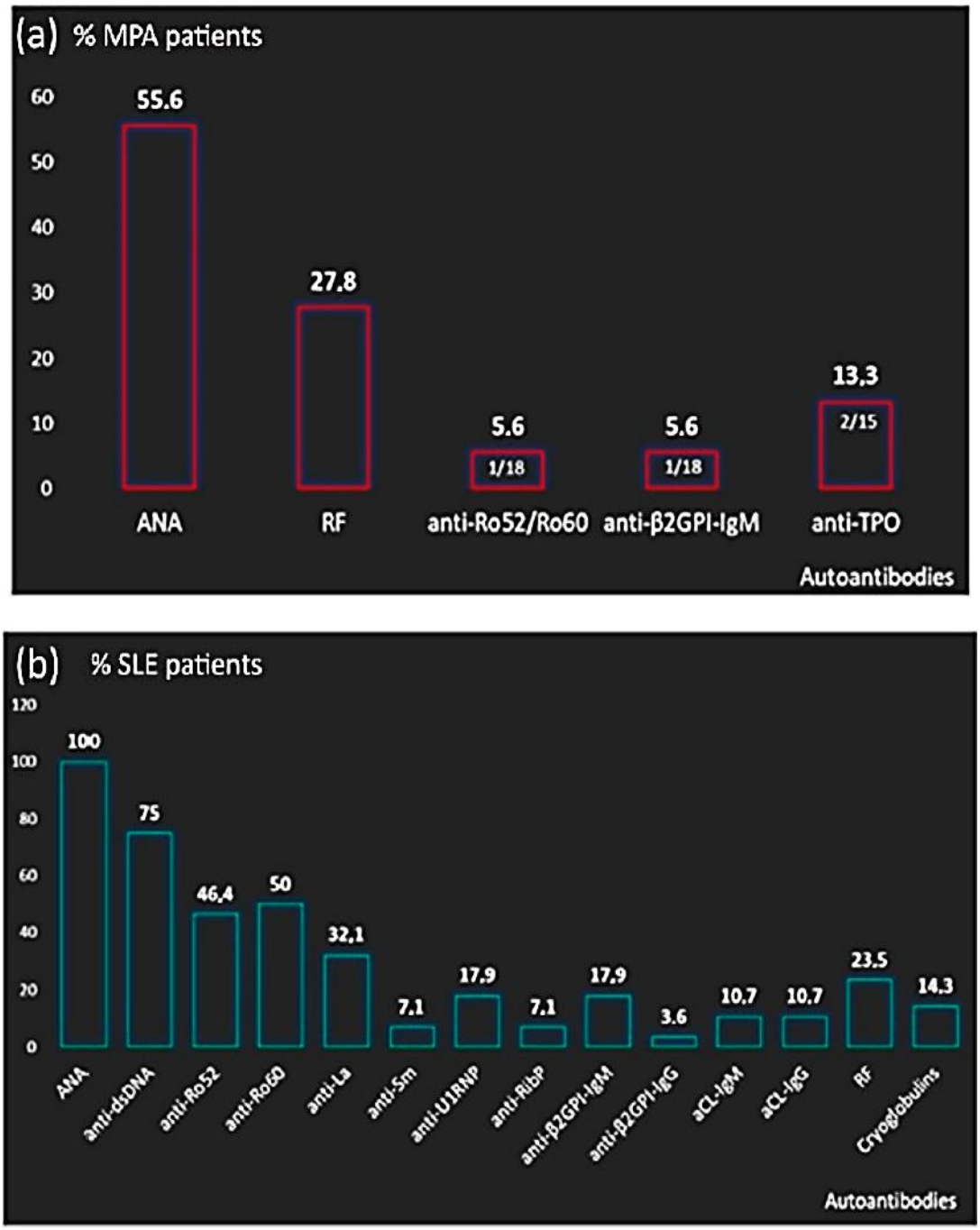

Figure 6.2 *Autoantibody profile of P-ANCA positive patients. (a) Presence other than P-ANCA autoantibodies in MPA patients; (b) distribution of autoantibodies in the context of SLE.*

6.4 Discussion

In this report, we investigated the occurrence of P-ANCA autoantibodies and their specificity in various autoimmune diseases. In accordance with previous studies (230), this study further indicates that MPO is the predominant autoantigen targeted by P-ANCA in MPA patients, whereas reactivity to other P-ANCA-related autoantigens, such as lactoferrin, may only sporadically be observed (258). Nevertheless, our study indicates that the antigenic specificity of P-ANCA autoantibodies remains elusive in a significant proportion of such patients. On the other hand, our results support that high titers of P-ANCA autoantibodies are frequently observed in patients with systemic autoimmune diseases, other than MPA. Interestingly, despite the relatively high P-ANCA titers, the vast majority of these patient groups have unidentified specificities. In line with previous reports (232,239,240,244), the most frequently recognized antigen in P-ANCA-positive patients is MPO, whereas reactivity against other P-ANCA-related autoantigens, such as lactoferrin and elastase, were rarely observed. Importantly, P-ANCA-positive SLE and MPA patients presented with enriched autoantibody profile, implying systemic autoimmune responses against ubiquitous self-antigens. Although the number of SLE P-ANCA positive patients is small, it is noteworthy that P-ANCA positivity among these patients is associated with skin, renal and hematologic manifestations. Interestingly, the anti-MPO positive lupus patient had no renal involvement, while anti-lactoferrin specificity was linked to the most severe clinical phenotype as opposed to double anti-MPO/lactoferrin specificity relayed to a milder clinical picture. These findings imply that P-ANCA specificity may have clinical significance; however, further multicentric studies of large patient cohorts are needed to verify these observations.

Important limitations of this study were the small number of patients, as well as the small number of autoantigens included in the assay we applied. A variety of potential self-antigens may be recognized by P-ANCA autoantibodies. Thus, diverse autoantigen array and/or high throughput biotechnologies are required to reveal potentially hidden specificities of P-ANCA reactivity. On the other hand, although limited, the misinterpretation of A-ANCA, as P-ANCA, cannot be excluded since our detection was based on immunofluorescence detection of only ethanol-fixed neutrophils. The

parallel use of ethanol and formaldehyde-fixed neutrophils for the detection of P-ANCA has been reported to discriminate false P-, atypical ANCA (259). However, this approach applies mainly for recognizing P-ANCA targeting MPO, whereas it has been reported to be non-beneficial for the study of SARDs patients other than those with vasculitides (259-261). Furthermore, the likelihood of the detection of P-ANCA-related pattern in the setting of concurrent high ANA titers cannot be excluded as ANA may produce indistinguishable immunofluorescent staining patterns on ethanol-fixed neutrophils. In fact, charge interactions between DNA and MPO may cause false positivity in MPO-ANCA in case of high anti-dsDNA sera titer (262). However, this possibility is significantly diminished by the fact that the detection of the characteristic perinuclear ring pattern is a prerequisite finding for the diagnosis of P-ANCA staining pattern in our departments.

In summary, P-ANCA are present in sera of patients with various systemic autoimmune diseases in high titers and are associated with ANA, confirming to most likely be in support of the systemic nature of autoimmunity. The results of this study indicate that, although MPO represents the most common P-ANCA specificity for MPA, autoantibodies to additional novel neutrophilic self-antigens are likely present in P-ANCA-positive autoimmune disease patients. In this context, further investigation of the currently unidentified P-ANCA-related autoantigens may reveal novel and clinically useful disease markers.

CHAPTER 7. CRYOGLOBULINEMIC VASCULITIS IN PRIMARY SJÖGREN'S SYNDROME: CLINICAL PRESENTATION, ASSOCIATION WITH LYMPHOMA AND COMPARISON WITH HEPATITIS C RELATED DISEASE.

7.1 Objectives

Cryoglobulinemic vasculitis is a life-threatening immune complex mediated small vessel vasculitis, involving primarily the skin, kidneys, and peripheral nerves, leading to end stage organ/tissue damage, if untreated (263,264). It can be of infectious origin, with HCV infection being the most common cause, or associated with autoimmune diseases where the most frequent underlying condition is pSS. As new, effective therapeutic modalities for the management of HCV infection keep emerging, pSS will soon become the leading cause of CV (265). CV is associated with the presence of serum cryoglobulins of which, type II containing an IgMk monoclonal rheumatoid factor (mRF) predominate in pSS, while both type II and III are detected in HCV and other autoimmune diseases. Previous studies have clearly shown that type II cryoglobulinemia in pSS is associated with more systemic manifestations, mainly vasculitis (131), and higher risk for future lymphoma development (132). Thus, cryoglobulinemia is at the crossroad of the two most serious complications of pSS, that is, systemic vasculitis and B-cell NHL. With the advent of new treatments, successfully targeting B-cells and NHL (266), the investigation and mapping of the clinical spectrum of pSS-CV may provide a concise strategic plan for early diagnosis and treatment for this subset of pSS. To this end, older studies have described the clinical picture of pSS patients in association with cryoglobulinemia (130,267,268), while after the introduction of CV criteria (162,269) some studies have focused on CV (138,270-273). However, even after the application of CV classification criteria, the clinical picture of pSS-CV is still obscured due to the fact that many of the previous studies have a small number of pSS patients.

Herein, we present the clinical phenotype of CV in an integrated study population of Greek-Italian pSS patients, investigate the possible role of CV and cryoglobulinemia in NHL development in pSS and compare the clinical manifestations of pSS-CV with HCV CV.

7.2 Patients and Methods

Study design

This is a retrospective, matched case-control study in a multicenter population of consecutive pSS patients who fulfilled the 2016 ACR/EULAR classification criteria (250) and were followed up from May 1984 until March 2019, in 5 centers from Greece and Italy (University of Udine, Pisa, Athens, Harokopio, Ioannina) (UPAHI group). The study was approved by the local ethical committees of all the Institutions involved, after obtaining patients' informed consent and in compliance to general data protection regulations (GDPR). One thousand eighty-three patients had been evaluated for serum cryoglobulins and 71 of them fulfilled the 2011 classification criteria for CV (162). Cryoglobulins were evaluated after blood collection, quantitation, immunodiffusion and immunofixation, as described previously (131). All pSS patients were HCV-RNA negative. The cumulative clinical, laboratory and histologic data of pSS-CV patients were compared with two control groups: a) pSS patients, repeatedly negative for serum cryoglobulins, matched (1:2 ratio) according to gender, age at pSS onset and disease duration from pSS onset and b) patients with HCV related CV, being RNA positive at CV diagnosis and without any associated autoimmune rheumatic disease, matched (1:1) according to age and gender. All patients with HCV related CV were diagnosed, treated and followed-up at the Infectious Disease Unit, Department of Clinical and Biomedical Sciences Hospita L. Sacco, Milan, Italy. In addition, pSS cryoglobulin-positive patients who did not fulfill the 2011 CV classification criteria, were compared with a pSS cryoglobulin negative control group, matched (1:2 ratio) according to gender, age, and disease duration from pSS onset. All the laboratory, objective tests, or minor salivary gland biopsy of pSS patients, were performed in the context of standard of care, according to physicians' judgment. pSS onset was defined as the year when the patient recalled the first disease related manifestation, such as Raynaud's phenomenon, arthritis, sicca symptoms, salivary gland enlargement or purpura. CV onset was defined as the time point of the appearance of the first CV related manifestation, according to the 2011 classification criteria. Groups were compared on the basis of cumulative clinical (dry mouth, dry eyes, salivary gland enlargement, Raynaud's phenomenon, lymphadenopathy, arthralgia/myalgia,

arthritis, palpable purpura, liver involvement, kidney involvement, central and peripheral nervous system involvement, lymphoma), laboratory (anti Ro/SSA antibodies, anti La/SSB antibodies, rheumatoid factor, cryoglobulinemia, low serum C4 complement levels, monoclonal gammopathy) and histologic (focus score, germinal centers) features. Systemic organ involvement was based on the ESSDAI definitions and/or biopsy specimens (274). Fatigue, dryness, and pain were assessed as defined by the ESSPRI (275). Since different pathogenetic mechanisms are operating in glandular and extraglandular manifestations of pSS, they have been classified as glandular (dry mouth, dry eyes, salivary gland swelling), non-specific manifestations (fatigue, arthralgia/myalgia, arthritis, Raynaud's phenomenon), peri-epithelial (interstitial nephritis, primary biliary cholangitis, small airways disease), immune complex mediated (extra-epithelial) (purpura, skin ulcers, glomerulonephritis, vasculitic involvement of peripheral and/or central nervous system) and NHL (103).

Statistical and data driven analysis

Statistical analysis for categorical data was performed by x2 test with Yates correction or Fisher exact when cell counts <5 patients and for numerical data t-test or Mann-Whitney, after Shapiro-Wilk normality test. In order to handle the multiple comparison testing, p-values have been also adjusted with Bonferroni correction. The Fast-Correlation based feature selection (FCBF) algorithm was applied on the dataset of pSS-CV patients and their pSS cryoglobulin negative controls, to identify potentially independent variables associated with lymphoma (276). The FCBF preselection algorithm is a correlation-based tool identifying, among several potentially independent variables, those with the weakest association amongst them and the strongest correlation with the outcome of interest that is NHL. Subsequently, the strongest preselected group of the FCBC derived potentially independent variables, has been used for constructing a binary multivariable logistic regression model to identify independent variables/features associated with lymphoma. The implementation of the FCBF-based multivariable logistic regression approach along with the statistical analysis was performed using Python 3.6 and GraphPad 7.0a. Based on the post hoc sample size and study power calculation conducted according to the Fleiss method, assuming 90% study power and 95% two-sided levels of confidence,

the present study sample size could detect an effect size (Odds Ratio) of 5.00 between patient groups (EpiInfo, CDC, Atlanta, Georgia, USA).

7.3 Results

Patient characteristics

Serum cryoglobulins were detected in 115/1083 patients (10.6%) of whom 71 (61.7%) fulfilled the 2011 CV classification criteria, while 44 (38.3%) had pSS with cryoglobulinemia but did not meet the CV criteria. Early (≤35 years) pSS onset had 19.7% (n = 14/71) of pSS-CV patients, while 12.7% (n = 9/71) had late (≥65 years) pSS onset. Among pSS-CV patients, 97% were females (n = 69/71) and 3% males (n = 2/71). The median age of pSS-CV patients, calculated at pSS onset, was 50 years (range: 21-75). The median duration from pSS onset in pSS-CV patients was 16 years (range: 0-37). None of the pSS or RNA HCV positive control patients fulfilled criteria for another systemic autoimmune disease. pSS cryoglobulin positive patients without vasculitis were also predominantly females [95.5%, (n = 42/44) vs 4.5%, (n = 2/44)] with a median age at pSS onset of 50 years (range:11-79) and median disease duration from pSS onset of 13 years (range: 0-42).

The clinical phenotype of pSS-cryoglobulin positive patients with and without CV

The clinical picture of 71 pSS-CV patients was compared with that of 141 pSS cryoglobulin negative matched control patients. pSS-CV patients exhibited higher frequency of fatigue (59.2% vs 43%, p= 0.041, OR = 1.92, 95% CI: 1.08-3.52), Raynaud's phenomenon (47.9% vs 32.6%, p = 0.044, OR = 1.89, 95% CI: 1.06-3.39), salivary gland enlargement (53.6% vs 33.3%, p = 0.007, OR = 2.31, 95% CI:1.29-4.21) and interstitial renal disease (10% vs 1.5%, p = 0.007, OR = 7.55, 95% CI: 1.59-36.4), compared to pSS-cryoglobulin negative patients respectively. No difference was found in sicca manifestations between the 2 groups. As anticipated, pSS-CV patients had increased prevalence of extra-epithelial manifestations of a vasculitic origin, including purpura (90.1% vs 14.9%, p < 0.0001, OR = 52.24, 95% CI: 21.43-125.4), vasculitic ulcers (12.7% vs 0.71%, p < 0.001, OR= 20.32, 95% CI:3.17-224.4), peripheral nervous system vasculitic involvement (25.4% vs 1.5%, p< 0.0001, OR = 21.74, 95% CI: 5.12-96.01),

glomerulonephritis, mainly of membranoproliferative type, (11.4% vs 0.71%, p< 0.001, OR = 18.06, 95% CI: 2.64-201.8) and lymphadenopathy (31% vs 7.1%, p < 0.001, OR = 5.88, 95% CI: 2.63-12.99). NHL, mainly of MALT type, was more frequent in the pSS-CV group (47.9% vs 8.5%, p < 0.0001, OR = 9.87, 95% CI: 4.7-20.9). Minor salivary gland biopsies of pSS-CV patients displayed a higher proportion of germinal centers (35% vs 11.3%, p = 0.043, OR = 4.21, 95% CI: 1.20-13.45) and a higher focus score [median: 2.05 (range: 0-9) vs 1.45 (range: 0-7)] compared to pSS cryoglobulin negative controls. The laboratory analysis disclosed that almost all pSS-CV patients had positive rheumatoid factor (95.7% vs 61.3, p < 0.0001, OR = 14.09, 95% CI: 4.58-44.33) as well as low C4 complement levels (88.6% vs 31.5, p< 0.0001, OR = 16.82, 95% CI: 7.47-35.57). Monoclonal gammopathy was also more prevalent in the pSS-CV group (45.5% vs 7.6%, p < 0.0001, OR = 10.17, 95% CI: 4.41-22.17). The presence of anti-Ro/SSA and anti-La/SSB autoantibodies was comparable between the two groups. A more detailed comparison of the clinical, laboratory, serological and histologic features between the 2 groups are presented in Table 7.1. After applying Bonferroni correction, fatigue, Raynaud's phenomenon, salivary gland enlargement, interstitial renal disease, focus score and germinal centers were found with no statistically significant difference between the 2 groups (Table 7.1).

	pSS-CV	pSS-Cryo negative	*P*-value	*P*-value*
Number of patients	71	141		
Gender (female%)(n)	97.2 (69/71)	96.5 (136/141)	1	1
Disease duration (from SS onset _years)	16 (range: 0–36)	15 (range: 0–34)	0.720	1
Media age (years)	50 (range: 21–75)	50 (range: 20–77)	0.887	1
Clinical Features% (n)				
Dry mouth	97.2 (69/71)	93.6 (132/141)	0.342	1
Dry eyes	98.6 (70/71)	92.9 (131/141)	0.104	1
SGE	53.6 (37/69)	33.3 (47/141)	0.007	0.252
Lymphadenopathy	31 (22/71)	7.1 (10/141)	<0.0001	<0.001
Fatigue	59.2 (42/71)	43(55/128)	0.041	1
Arthralgia/myalgia	71.8 (51/71)	63.8 (90/141)	0.312	1
Arthritis	25.7 (18/70)	18.3 (23/126)	0.295	1
Myositis	0(0/71)	1.4 (2/141)	0.552	1
Raynaud's phenomenon	47.9 (34/71)	32.6 (46/141)	0.044	1
Purpura	90.1 (64/71)	14.9 (21/141)	<0.0001	<0.0001
Vasculitic ulcer	12.7 (9/71)	0.71 (1/141)	<0.001	0.009
PNS	25.4 (18/71)	1.5 (2/130)	<0.0001	<0.0001
CNS	2.8 (2/71)	2.3 (3/129)	1	1
Liver				
Sclerosing Cholangitis	0(0/71)	0(0/141)	1	1
AIH	1.4 (1/71)	1.4 (2/141)	1	1
PBC	1.4 (1/71)	2.8 (4/141)	0.666	1
Lung				
ILD	8.6 (6/70)	6.8 (9/133)	0.853	1
Small airways disease	13(7/54)	6.6 (8/121)	0.273	1
Kidney				
Interstitial renal disease	10 (7/70)	1.5 (2/138)	0.007	1
Glomerulonephritis	11.4 (8/70)	0.71 (1/141)	<0.001	0.026
Splenomegaly	2.8 (2/71)	10.8 (8/74)	0.260	1
Lymphoma	47.9 (34/71)	8.5 (12/141)	<0.0001	<0.0001
Biological features% (n)				
Focus score (median)	2.05 (34/71)	1.42 (78/141)	0.025	0.9
Germinal centers	35 (7/20)	11.3 (6/53)	0.043	1
Anti-Ro	90 (63/70)	85(119/140)	0.429	1
Anti-La	60 (42/70)	46(64/139)	0.078	1
Anti-Ro/La	88.4 (61/69)	83.6 (117/140)	0.472	1
RF	95.7 (67/70)	61.3 (84/137)	<0.0001	<0.0001
Low C4	88.6 (62/70)	31.5 (41/130)	<0.0001	<0.0001
Hypergammaglobulinemia	69.7 (46/66)	56.9 (74/130)	0.114	1
Monoclonal gammopathy	45.5 (30/66)	7.6 (10/132)	<0.0001	<0.0001
Leukopenia	17.4 (12/69)	10 (14/140)	0.193	1
Thrombocytopenia	3(2/67)	2.2 (3/134)	1	1

Table 7.1. *Comparison of clinical, laboratory and histologic features between pSS-CV and pSS patients with negative serum cryoglobulins.*

The FCBF algorithm, that constitutes the data driven analytic approach, performed analysis of 36 features/variables and disclosed 8 strong potentially independent variables [lymphadenopathy, cryoglobulinemia, positive RF, SGE, dry mouth, arthritis, CNS involvement and lungbronchocentric involvement accounting for small airways disease]. Narrowing of the analysis, using a combined FCBF/multivariable logistic regression model, showed that lymphadenopathy, cryoglobulinemia and positive RF are independent lymphoma associated features. The performance of the model disclosed 70.6% sensitivity, 94.7% specificity and 84.5% accuracy area under the curve (AUC = 85.5%), after a 10-fold cross validation approach.

The most common presenting manifestations, according to the 2011 CV classification criteria, of pSS-CV patients were arthralgia/myalgia (49%), followed by arthritis (43.7%) and purpura (39.1%) (Figure 7.1A). More than half of the patients (58.2%) with CV developed the first CV specific manifestation within the first year of pSS onset (Figure 7.1B). In one third of pSS-CV patients (33.3%), lymphoma was observed within 5 years of CV onset, while in 13.3% of them, the appearance of lymphoma was a late sequel (>15 years of CV duration) (Figure 7.1C).

The comparison between pSS cryoglobulin positive patients without CV and pSS cryoglobulin negative controls is presented in Table 7.2. The pSS cryoglobulin positive group without CV, had a higher frequency of monoclonal gammopathy (22% vs 7.3%, p= 0.040, OR = 3.56, 95% CI: 1.16-11.16) and NHL (29.5% VS 5.9%, P<0.001, OR = 6.26, 95% CI: 2.13-17.67). Data regarding lymphoma and type of cryoglobulins were available in 27 of 44 pSS cryoglobulin positive patients without CV. Type II cryoglobulinemia, containing an IgMk monoclonal RF, was present in 87.5% (n= 7/8) of pSS patients with lymphoma and 47.3% (n = 9/19) in those without, while type III cryoglobulinemia was more prevalent in non-lymphoma (52.7%, n= 10/19) compared to lymphoma (12.5%, n = 1/8) pSS patients. After Bonferroni adjustment, monoclonal gammopathy was found with no statistically significant difference between the 2 groups (Table 7.2).

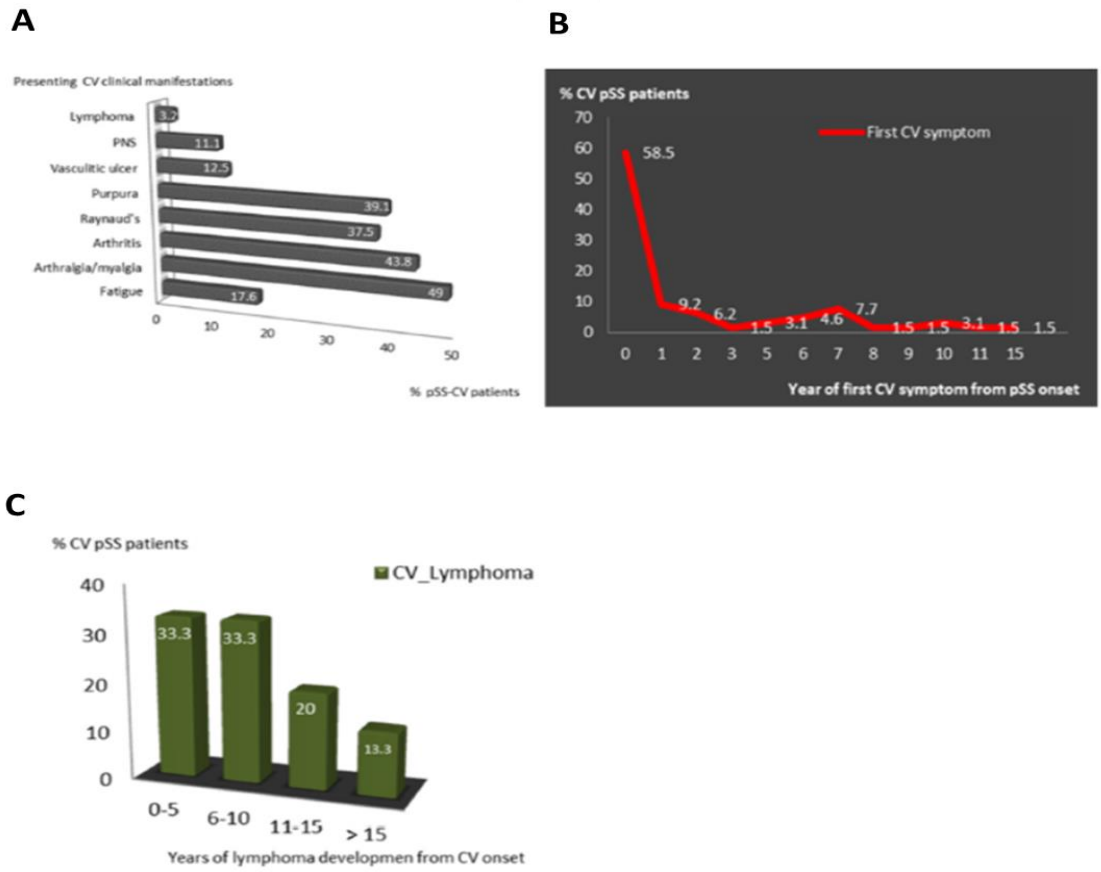

Figure 7.1. *The clinical course of pSS-CV patients. 1A) Most common presenting clinical manifestations of CV after applying the 2011 CV classification criteria, 1B) Chronological presentation of first CV related manifestation after pSS onset, 1C) Time distribution of lymphoma development during the course of CV.*

Overall, the 71 pSS-CV patients with either mild/non-specific or serious manifestations were treated as follows: GCs 86%, HCQ 80.3%, AZA 21.1%, MTX 22.5%, CYC 8.5%, RTX 18.3% and plasmapheresis 7%. Notably, pSS-CV patients without lymphoma received more frequently AZA, MTX and CyC compared to those with lymphoma (32.4% vs 8.8%, 27% vs 17.6% and 10.8% vs 5.9 respectively). The 141 pSS cryoglobulin negative controls have received: GCs 31.4%, HCQ 46.4%, AZA 4.3%, MTX 7.1%, CyC 0.7% and RTX 4.3%. Interestingly, pSS-CV patients with serious manifestations such as glomerulonephritis, peripheral neuropathy and vasculitic ulcers were treated with steroids, hydroxychloroquine, B cell depletion therapy and very few cases with cyclophosphamide or plasmapheresis. The therapeutic regimen of the 44 cryoglobulin positive without CV pSS patients versus their controls included: GCs 38.6% vs 39%,

HCQ 45.5% vs 65.9%, AZA 9.1% vs 6.1%, MTX 2.3% vs 7.3%, CyC 4.5% vs 2.4% and RTX 4.5% vs 8.5%.

	pSS-Cryo without CV	pSS-Cryo negative	*P*-value	*P*-value*
Number of patients	44	84		
Gender (female)% (n)	95.5 (42/44)	95.2 (80/84)	0.700	1
Median age (years)	50 (range:11–79)	51	0.695	1
Median disease duration (from SS onset _years)	13 (range:0–42)	11.5	0.628	1
Clinical features% (n)				
Dry mouth	95.5 (42/44)	95.2 (80/84)	1	1
Dry eyes	97.7 (43/44)	95.2 (80/84)	0.659	1
SGE	48.8 (21/43)	29.8 (25/84)	0.054	1
Lymphadenopathy	13.6 (6/44)	6 (5/84)	0.253	1
Fatigue	36.8 (14/38)	43.6 (37/80)	0.444	1
Arthralgia/myalgia	56.8 (25/44)	53.6 (45/84)	0.870	1
Arthritis	10.8 (4/38)	8.9 (7/79)	0.746	1
Myositis	0 (0/44)	0 (0/84)	1	1
Raynaud's phenomenon	38.6 (17/44)	28.6 (24/84)	0.337	1
Purpura	6.8 (3/44)	15.5 (13/84)	0.253	1
Vasculitic ulcer	2.3 (1/44)	1.2 (1/84)	1	1
PNS	4.5 (2/44)	3.6 (3/82)	1	1
CNS	2.3 (1/44)	2.4 (2/82)	1	1
Liver				
Sclerosing Cholangitis	0 (0/44)	2.4 (2/784)	0.545	1
AIH	0 (0/44)	0 (0/84)	1	1
PBC	0(0/42)	3.6 (3/84)	0.550	1
Lung				
ILD	9.1 (4/44)	4.9 (4/81)	0.450	1
Small airways disease	0 (0/40)	6.5 (5/77)	0.163	1
Kidney				
Interstitial renal disease	4.5 (2/44)	1.2 (1/83)	0.275	1
Glomerulonephritis	2.3 (1/44)	1.2 (1/83)	1	1
Splenomegaly	0 (0/44)	0 (0/84)	1	1
Lymphoma	29.5 (13/44)	6(5/84)	<0.001	0.023
Biological features% (n)				
Anti-Ro/SSA	70.5 (31/44)	71.4 (60/84)	0.928	1
Anti-La/SSB	25 (11/44)	41.7 (35/84)	0.094	1
RF	70.7 (29/41)	53(44/83)	0.090	1
Low C4	53.6 (22/41)	36.3 (29/80)	0.100	1
Hypergammaglobulinemia	59(23/39)	55(44/80)	0.831	1
Monoclonal gammopathy	22 (9/41)	7.3 (6/82)	0.040	1
Leukopenia	4.5 (2/44)	8.3 (7/84)	0.717	1
			0.122	1

Table 7.2 *Clinical, laboratory and histologic features of cryoglobulin positive pSS patients without CV compared to pSS cryoglobulin negative patients.*

Comparison of pSS-CV vs HCV-CV patients

The differences between the 71 pSS-CV and the 76 HCV-CV matched patients are presented in Table 12. As expected, dry eyes (98.6% vs 21.1%, $p < 0.0001$, OR = 262.5, 95% CI: 42.14-2680), dry mouth (97.2% vs 18.4%, $p < 0.001$, OR = 152.8, 95% CI: 34.41657.8) and salivary gland enlargement (53.6% vs 0%, $p< 0.0001$) were more frequent among pSS-CV patients compared to HCV-CV. In addition, lymphadenopathy (31% vs 4%, $p < 0.001$, OR = 10.78, 95% CI:3.29-35.17), arthritis (25.7% vs 10.8%, p= 0.035, OR = 2.85, 95% CI:1.17-6.86), Raynaud's phenomenon (47.9% vs 22.4%, p= 0.002, OR = 3.18, 95% CI: 1.59-6.45), interstitial renal disease (10% vs 1.4%, p= 0.030, OR = 8.11, 95% CI: 1.36-92.47), hypergammaglobulinemia (69.7% vs 26.2%, $p < 0.001$, OR = 6.49, 95% CI: 2.98-13.41) and type II IgMk cryoglobulinemia (97% vs 80.3%, p = 0.035, OR = 7.86, 95% CI:1.15-85.55) were more often observed in pSS-CV compared to HCV-CV patients. On the contrary, HCV infection was associated with increased frequency of fatigue (98.6% vs 59.2%, $p< 0.0001$, OR = 47.64, 95% CI: 8.18495.9), peripheral nervous system vasculitic involvement (71.6% vs 25.4%, $p< 0.0001$, OR= 7.43, 95% CI: 3.59-15.57) and low C4 (98.5% vs 88.6%, p = 0.033, OR = 8.51, 95% CI: 1.22-95.82), compared to pSS. The prevalence of lymphoma development was significantly higher in pSS-CV compared to HCV-CV patients (47.9% vs 13%, $p < 0.0001$, OR = 6.12, 95% CI: 2.7-14.4). After Bonferroni correction, arthritis, Raynaud's phenomenon, interstitial renal disease, low C4 and type II cryoglobulinemia were found with no statistically significant difference between the 2 groups (Table 7.3).

	pSS-CV	HCV-CV	*P*-value	*P*-value*
Number of patients	71	76		
Gender (female)% (n)	97.2 (69/71)	97.4 (74/76)	1	1
Median age (years)	50 (range: 21–75)	51 (range: 29–62)	0.718	1
Clinical Features% (n)				
Dry mouth	97.2 (69/71)	18.4 (14/76)	<0.0001	<0.0001
Dry eyes	98.6 (70/71)	21.1 (16/76)	<0.0001	<0.0001
SGE	53.6 (37/69)	0(0/75)	<0.0001	<0.001
Lymphadenopathy	31 (22/71)	4(3/75)	<0.0001	<0.001
Fatigue	59.2 (42/71)	98.6 (69/70)	<0.0001	<0.0001
Arthralgia/myalgia	71.8 (51/71)	73.7 (56/76)	0.797	1
Arthritis	25.7 (18/70)	10.8 (8/74)	0.035	1
Myositis	0(0/71)	0(0/75)	1	1
Raynaud's phenomenon	47.9 (34/71)	22.4 (17/76)	0.002	0.062
Purpura	90.1 (64/71)	88.2 (67/76)	0.902	1
Vasculitic ulcer	12.7 (9/71)	25(19/76)	0.090	1
PNS	25.4 (18/71)	71.6 (53/74)	<0.0001	<0.0001
CNS	2.8 (2/71)	1.4 (1/71)	1	1
Liver				
Sclerosing Cholangitis	0(0/71)	0(0/76)	1	1
AIH	1.4 (1/71)	0(0/70)	1	1
PBC	1.4 (1/71)	0(0/72)	0.496	1
Lung				
ILD	8.6 (6/70)	1.3 (1/75)	0.056	1
Small airways disease	13 (7/54)	4(3/75)	0.093	1
Kidney				
Interstitial renal disease	10 (7/70)	1.4 (1/74)	0.030	0.93
Glomerulonephritis	11.4 (8/70)	23.7 (18/76)	0.085	1
Splenomegaly	2.8 (2/71)	10.8 (8/74)	0.097	1
Lymphoma	47.9 (34/71)	13(9/69)	<0.0001	<0.001
Biological features% (n)				
RF	95.7 (67/70)	87.1 (61/70)	0.128	1
Low C4	88.6 (62/70)	98.5 (66/67)	0.033	1
Cryoglobulinemia type II	97 (32/33)	80.3 (61/76)	0.035	1
Hypergammaglobulinemia	69.7 (46/66)	26.2 (17/65)	<0.0001	<0.0001
Monoclonal gammopathy	45.5 (30/66)	55.5 (20/36)	0.442	1
Leukopenia	17.4 (12/69)	14.7 (10/68)	0.845	1
Thrombocytopenia	3(2/67)	11.4 (8/70)	0.097	1

Table 7.3 *Comparison of the clinical, laboratory and histologic features between pSS-CV and HCV-CV patients.*

7.4 Discussion

Cryoglobulinemic vasculitis is a rare disease presenting in approximately 4-11% of pSS patients (138,277,278). Despite the rarity, the disease exhibits two major clinical elements leading to poor outcome in pSS, which are systemic vasculitis and lymphoma development. This information has been concluded by several case and cohort studies in the past (130,138,278-280). However, the major determining factor of those studies was the presence of cryoglobulinemia while more precise data according to the international and validated classification criteria for CV are limited (138,162,231,272,281,282). Thus, the clinical presentation of pSS-CV, as well as the characteristics that rule the outcome and the definition of the clinical phenotype of pSS-CV are still unmet needs.

The present study was conducted to address the clinical presentation of CV in unselected patients with pSS. The tools that were used include: a) an integrated Greek-Italian population, from 5 clinical centers evaluated by physicians, highly experienced with pSS-CV, b) application of unified and validated criteria for both pSS and CV, c) carefully selected triple matched 1:2 controls from the same clinical center, and d) analysis of the results by applying not only the classic statistics, but beyond that, a data driven approach with an unbiased selection of variables, to point out features associated with CV which are involved in lymphoma development. HCV infection often mimics pSS, sharing common clinical manifestations, including sicca symptoms and CV. To address this challenging diagnostic question, we compared the clinical picture of pSS-CV with that of HCV-CV.

To our knowledge this is the largest study of pSS-CV patients fulfilling the 2011 CV classification criteria. The prevalence of cryoglobulins is within the range reported by previous studies, (130,138,267,268,272) corresponding to 10% of the evaluated pSS patients. The clinical picture described in this report is in line with previous studies, but some points are presented for the first time and deserve special attention: a) approximately 60% of pSS-CV patients had their first CV manifestation within the first year from pSS onset, b) pSS-CV is heralded by non-specific clinical manifestations, such as arthralgia/myalgia, arthritis or Raynaud's phenomenon, c) high prevalence of skin vasculitis, extending from the one third of patients at disease onset to almost all

patients after many years of follow up and d) a time-related pattern for the appearance of glomerulonephritis and peripheral neuropathy was not observed. One-third of the associated NHL cases occurred during the first 5 years after CV onset, but the diagnosis of NHL for the majority of pSS-CV patients, spread out in 20 years, since CV diagnosis. Based on these observations, since pSS-CV is usually heralded by non-specific manifestations of pSS, it is strongly recommended that, pSS patients must be evaluated properly at pSS onset for the presence of cryoglobulins. Following early diagnosis, pSS-CV patients should undergo a close follow up for many years, since internal organ vasculitis or lymphoma can very well be late sequels. These findings can also start a discussion, whether early intervention with targeted B-cell treatments, might be instituted after the detection of cryoglobulins, even if this is associated with non-specific symptoms, in an attempt to prevent overt vasculitis and/or lymphoma. Finally, the present study has clearly shown that pSS-CV and HCV-CV, are distinct entities, since pSS-CV patients present much more frequently with sicca manifestations, lymphadenopathy, arthritis and lymphoma, mainly in the context of type II cryoglobulinemia with an IgMk mRF (283).

In accordance with previous reports (120,138,281), purpura is the most prevalent CV-specific manifestation of pSS-CV. This study, adds that purpura is also the most common CV-specific presenting manifestation occurring in one-third of patients within the first year after pSS onset, thus explaining why purpura at pSS diagnosis is an excellent predictor for future lymphoma development (284). Although the clinical expression of pSS-CV is spreading across time, this study showed a temporal clustering of CV and pSS onset in 60% of pSS-CV patients. In line with this, one third of pSS-CV will eventually develop a NHL of B cell origin within the first 5 years of pSS onset. Taken together, it appears that this subset of pSS patients possess a discrete B cell monoclonal population, producing an IgMk monoclonal RF, very early during pSS disease course. Importantly, previous data point out that the monoclonal component is composed within the salivary glands (285,286), explaining why SGE in several reports is an independent predictor of future lymphoma development. The production and perpetuation of mRFs within the inflamed salivary glands are probably attributed to the formation of EGCS, which are more frequently found in pSS-CV patients compared to cryoglobulin negative controls. On the contrary, in HCV-related cryoglobulinemia

(270), B-cell clonal expansion is mainly localized within the bone marrow and the liver (287). Compared to HCV-CV, pSS-CV patients carry an increased risk of lymphoma that could be explained by the occurrence of MALT lymphomas unrelated to cryoglobulinemia (270) and by the more intense and persistent autoreactive B cell activation as attested by the higher frequency of lymphadenopathy, hypergammaglobulinemia and specific autoantibodies, observed in pSS-CV patients. On the other hand, HCV-CV patients had a remarkably increased peripheral nervous system involvement which could be attributed to 2 distinct underlying mechanisms: a) inflammation of the vasa nervorum not only due to cryoglobulins but also to anti-HCV/HCV immune complex deposition, and b) direct HCV mediated inflammation of the nerves (288-290)

Previous studies have clearly shown that cryoglobulinemia is associated with increased morbidity and mortality, serving also as one of the strongest laboratory predictors for future lymphoma development, either with or without CV (271,284,291). The answer to the question whether cryoglobulinemia or CV serves better as lymphoma risk factor is still unaddressed. Indeed, previous studies, using small number of patients, showed that neither CV nor cryoglobulinemia were proven risk factors for lymphoma in the multivariate model, although CV was correlated with increased mortality (138). In our dataset, we performed a data driven analysis to identify lymphoma associated features including both cryoglobulinemia and the presence of CV. Although, cryoglobulinemia was emerged as an independent feature, this finding is random, since in our specific dataset both cryoglobulinemia and CV possess the same power of significance, according to the FCBC algorithm and, eventually one of them, but not both could be selected as a potentially independent variable. The FCBF algorithm categorizes on a mathematical based manner, features and variables with minimal inter-correlation and therefore it was unexpected to pre-select both cryoglobulinemia and CV as potentially independent variables for the logistic regression model. Thus, it was highly unlikely to compare cryoglobulinemia and CV as independent variables for any type of LR model, since the first variable is a prerequisite for the second. Finally, to address this question, we analyzed concomitantly the group of the 44 pSS cryoglobulin positive patients without vasculitic tissue damage and compared it with the 71 pSS CV patients. Around half of the pSS-CV patients had NHL, while that was true only for one fourth of

cryoglobulin positive CV negative pSS patients. In the latter group the determining factor for lymphoma development was the presence of mRF in the cryoprecipitate (87.5% of patients with lymphoma) i.e. type II cryoglobulinemia and not type III. In the pSS-CV patients where the prevalence of lymphoma was double, the presence of IgMk mRF type II cryoglobulinemia was 97%. At this point, we feel it is important to emphasize that the capacity of serum cryoglobulins to precipitate in tissues may be affected by many factors: a) the quantity of circulating cryoglobulins as a net balance between production and clearance (292,293), b) the degree of the affinity-avidity of the monoclonal component with rheumatoid factor activity against the polyclonal IgG component (294), c) the physicochemical properties of cryoglobulins such as sialylation of the Fc portion and d) several environmental factors including temperature, pH and the presence of plasma hyperfiltration conditions in specific tissues (294,296). Thus, the cryoprecipitable IgMk mRF, the common denominator between the two groups, operates as either a double or single sword edge in patients with pSS-CV and pSS with cryoglobulinemia only, regarding lymphoma development. In the second case, it represents only the B-cell clonal expansion, whilst in the first case it is the major element responsible for the generation of the complex disease of CV that can be seen as a composite index, born by nature. This can explain why in several previous studies, many items of CV served as predictive factors for future lymphoma development. Another issue that remains inadequately addressed in the literature, is the net effect of specific treatment modalities on lymphoma development. In this series of pSS-CV patients, the majority of those with severe vasculitic involvement were treated with corticosteroids, hydroxychloroquine, B cell depletion therapy (rituximab) and plasmapheresis. In very few cases cyclophosphamide was administered, while MTX and AZA were more commonly used in pSS-CV patients compared to cryoglobulin negative controls. pSS-CV patients without lymphoma have been also treated more frequently with MTX and AZA compared to those with lymphoma. Therefore, it seems that the additional risk from the excess use of MTX and AZA among pSS-CV patients compared to controls is low, suggesting that systemic immunosuppression confers low risk to lymphoma development.

In this work, we present our results with and without Bonferroni correction that represents one of the most widely used methods for p-values adjustment to avoid type I error in the context of multiple comparisons. However, it has been supported that Bonferroni correction is very stringent and augments the occurrence of type II error (297). Therefore, many researchers including the authors of this manuscript, choose not to make any adjustments for multiple comparisons and present data on the original form (297), while part of the scientific community believes that Bonferroni correction is of limited use in biomedical science (298). For all reasons mentioned above, we show the original and Bonferroni adjusted p-values, adopting in this way all scientific opinions. At the end, CV was proven a major risk factor for lymphoma development. The statistical significance of this element was not lost after Bonferroni correction, a finding that further strengthens our conclusions. On the other hand, the non-specific features of CV such as Raynaud's phenomenon and fatigue did not retain statistical significance after adjustment, implying that a type II error may have occurred.

The current study has some limitations. Even if the group of pSS-CV patients is the largest ever described, its number is still relatively small and therefore, a larger group would allow a better power analysis. While our study was amply powered, the resulting confidence intervals of effect estimates, support the necessity for the refinement of exact effect size estimates, in further prospective investigations. The fact that not all pSS patients included in the total study population have been evaluated for cryoglobulins, probably underestimates the real prevalence of both cryoglobulinemia and CV, pointing out a selection bias. However, the evaluated pSS patients represent more serious cases and therefore, our results can be generalized, as they reflect the real clinical practice. In addition, the inclusion of non-specific manifestations in the 2011 classification criteria of CV is an inner deficit, leading to overestimation of such manifestations in the context of pSS CV, since they can be attributed to either the CV or pSS itself. Such limitations, regarding not only the clinical phenotyping of CV in pSS but also the need for validation, are expected to be overcome through a large multicenter study population in the context of the HarmonicSS project. Another limitation is the heterogeneity in terms of ethnicity, genetic background, and environmental influence between Greek and Italian pSS patients who participated in

the study. Finally, the FCBF/LR data driven analysis was applied on the dataset of pSS-CV and cryoglobulin negative patients, of whom 46 had lymphoma, a relatively small number that is anticipated to affect the sensitivity level of the model.

In summary, CV associated with pSS constitutes a specific clinical phenotype of pSS, associated with both an inflammatory component, clinically expressed as vasculitis and NHL development. The presence and type of cryoglobulins should be evaluated early and during follow up in every patient with pSS. In the majority of patients, CV is appeared early after pSS onset, with non-specific clinical manifestations or purpura. The major determining factor of CV is the IgMk mRF of type II mixed cryoglobulinemia, offering the opportunity to use B cell targeted treatments that have the potential to halt the progress of the disease. Finally, the clinical expression of pSS-CV, has certain differences compared to HCV related CV, reflecting biologic differences which may guide the physicians in the differential diagnosis and the proper therapeutic interventions.

Abstract

Systemic vasculitides constitute an heterogenous group of rare, chronic and often recurrent systemic autoimmune/autoinflammatory diseases, characterized by multi-level heterogeneity in terms of clinical phenotype, histologic patterns, pathogenetic mechanisms and treatment selection strategies. The vascular damage implicates various disease-specific mechanisms including natural and specific immunity, immune complex formation and presence ANCAs. The acceleration of three classical types of arterial damage (inappropriate arterial remodeling, atheromatosis and arteriosclerosis), affecting both the micro- and macro-circulation, has also been proposed as an additional mechanism of vascular injury at least in some PSV types. This is attributed to the interplay between tissue and systemic inflammation, immunosuppressive therapy, and common CVD risk factors and may contribute to the increased CVD morbidity and mortality of PSV patients. The identification of the variable clinical phototypes even within the same type of SV, the identification of clinically relevant biomarkers, concerning both the inflammatory process and comorbidities as well as the molecular stratification of patients towards precise targeted treatments are still unmet needs.

The present study aims to:

I. Explore the presence and potential reversibility of subclinical vascular dysfunction and/or damage both in the micro- and macro-circulation in SV by evaluating four main vascular pathologies (atheromatosis, arterial stiffening, arterial remodeling/hypertrophy and pressure wave reflection impairment) in four different vascular beds (carotid, aortic, femoral and retinal) using gold-standard in clinical practice, non-invasive vascular biomarkers. The monitoring of subclinical vascular damage might provide insights on the development of these vascular pathologies in SV as well as guide the management of these patients, in similar ways as in individuals with CVD risk factors.
II. To identify and investigate the specificity of already existing biomarkers such as pANCAs as well as the identification of novel biomarkers within the metabolic profile of patients related to disease activity.

III. To describe the clinical spectrum of cryoglobulinemic vasculitis in primary Sjögren's syndrome, identify the biomarkers associated with future lymphoma development and depict the differences with hepatitis C virus related CV.

Patients and Methods

A. To evaluate the micro- and macro-circulation in SV, 73 PSV patients, matched at 1:1 according to age/sex/CVD risk factors with non-inflammatory controls-(NIC) and rheumatoid arthritis-(RA) controls were studied. In the case of GCA, patients with PMR without underlying vasculitis served as a second disease control group. Atheromatosis-(carotid/femoral plaques), arterial stiffening-(cfPWV), pressure wave reflections-(AIx and AIx75) and arterial remodeling-(cIMT) and retinal vessel calibers (CRAE, CRVE) were evaluated in both active and inactive disease state.

B. To explore the metabolic profile in sequential sera of GCA and PMR patients 110 serum samples from 50 patients (33-GCA and 17 PMR) at 3 time points, 0-(V1: active disease), 1 and 6 months-(V2 and V3: remission) of treatment with glucocorticosteroids, were subjected to NMR-based metabolomic analysis. Multi- and univariate statistical analyses were utilized to unveil metabolome alterations following treatment. Moreover, we investigated the occurrence of ANCA-related antigenic specificities in 82 P-ANCA-positive sera by multiplex ELISA, as well as their association with other autoantibodies. The P-ANCA-positive sera corresponded to patients with SV (n=24), SLE (n=28), APS (n=5), SS (n=7), RA (n=3), SSCL (n=1), sarcoidosis (n=1) and Hashimoto thyroiditis (n= 13).

C. From 1083 pSS patients we identified 71 with cryoglobulinemic vasculitis. pSS-CV patients were matched with pSS patients without cryoglobulins (1:2) and HCV-CV patients (1:1). Clinical, laboratory and outcome features were analyzed. A data driven logistic regression model was applied for pSS-CV patients and their pSS cryoglobulin negative controls to identify independent features associated with lymphoma.

Results

I. Aortic PWV in PSV was higher by 0.7 m/sec compared to NIC, and by 1.3 m/sec to RA-controls-(p=0.003) and was more pronounced in LVV/MVV (p=0.08 and p=0.001

respectively). AIx was decreased in all PSV (p=0.03) and predominantly in SVV compared to NIC-(p=0.04) and RA-controls (p=0.09 all, p=0.07 active disease). Atherosclerotic plaque formation prevailed in all vascular beds at diagnosis being more enhanced in LVV/MVV (p=0.007, p=0.03, p=0.004), compared to NIC and only both carotid/femoral-(p=0.02) to RA-controls. Carotid IMT was higher in LVV/MVV irrespectively to disease state and control group and was the most sensitive to change biomarker between activity-inactivity. Active GCA was associated with increased PWV/plaques/cIMT compared to matched-NIC, RA and PMR-controls. Venular and arteriolar retinal dilatation was observed in all active disease groups, while inactivity was associated with irreversible dilatation of the arterioles compared to NIC-(p=0.029) and RA-controls-(p=0.008). In brief we found that non-invasive vascular biomarkers allow early identification of vascular pathology at disease diagnosis suggesting disease specific rather than treatment related CVD association, suggesting that early management of CVD risk factors as proposed by biomarkers assessing subclinical atheromatosis and arteriosclerosis could prevent long term CVD events in PSV patients.

II. Distinct metabolic profiles were identified between activity and remission, independently to disease type. N-acetylglycoproteins and cholines of bound phospholipids, emerged as predictive markers of disease activity. Altered levels of 4 out of the 21 small molecules were also observed, including increased levels of phenylalanine, and decreased of glutamine, alanine, and creatinine in active disease. Metabolic fingerprinting discriminated GCA from PMR in remission. GCA and PMR patients exhibited characteristic lipid alterations as a response and/or adverse effect of GCs treatment. Correlation analysis showed that several identified biomarkers were further associated with acute phase reactants, C-Reactive Protein and Erythrocyte Sedimentation Rate. The NMR profile of serum metabolome could identify and propose sensitive biomarkers of inflammation. Metabolome alterations, following GCs treatment, could provide predictors for future steroid-induced side effects.

III. In most P-ANCA-positive patients studied (51/82, 62.3%), these autoantibodies occurred in high titers (>1:160). The analysis of P-ANCA-positive sera revealed reactivity to MPO in only 50% of patients with vasculitides, whereas it was

infrequent in the other disease groups studied. Reactivity to other P-ANCA-related autoantigens was also rarely detected. Our findings support that high P-ANCA titers occur in SARD. The P-ANCA-positive staining pattern is associated with MPO specificity in vasculitides, while in other autoimmune diseases, it mostly involves unknown autoantigens.

IV. pSS-CV patients had higher frequency of extraglandular manifestations and lymphoma (OR=9.87, 95% CI: 4.7-20.9) compared to pSS patients without cryoglobulins. Purpura was the commonest vasculitic manifestation (90%), presenting at disease onset in 39% of patients. One third of pSS-CV patients developed B-cell lymphoma within the first 5 years of CV course, with cryoglobulinemia being the strongest independent lymphoma associated feature. Compared to HCV-CV patients, pSS-CV individuals displayed more frequently lymphadenopathy, type II IgMk cryoglobulins and lymphoma (OR = 6.12, 95% CI: 2.7-14.4) and less frequently C4 hypocomplementemia and peripheral neuropathy. In conclusion, pSS-CV has a severe clinical course, overshadowing the typical clinical manifestations of pSS and higher risk for early lymphoma development compared to HCV related CV. Though infrequent, pSS-CV constitutes a distinct severe clinical phenotype of pSS.

Future studies with long term follow-up and stratification according to immunosuppressive treatment will facilitate our better understanding of the link between vascular pathology and inflammation, yet providing new diagnostic, prevention and response treatment biomarkers.

Περίληψη

Οι συστηματικές αγγειίτιδες (ΣΑ) συνιστούν μια ετερογενή ομάδα σπάνιων, χρόνιων, με συχνές υποτροπές, ομάδα συστηματικών αυτοάνοσων/αυτοφλεγμονωδών νοσημάτων, που χαρακτηρίζονται από πολύ-επίπεδη ετερογένεια η οποία αφορά τον κλινικό φαινότυπο, τα ιστολογικά πρότυπα, τους παθογενετικούς μηχανισμούς και τις στρατηγικές επιλογής θεραπείας. Η αγγειακή βλάβη αποδίδεται σε διάφορους μηχανισμούς ειδικούς για τη νόσο, συμπεριλαμβανομένης της κυτταρικής ανοσίας, του σχηματισμού ανοσοσυμπλεγμάτων και της παρουσίας ANCA. Η επιτάχυνση των 3 κλασικών τύπων αρτηριακής βλάβης (αρτηριακή αναδιαμόρφωση, αθηρωμάτωση και αρτηριοσκλήρωση), που επηρεάζουν τόσο τη μικρο- όσο και τη μακρο-κυκλοφορία, έχει επίσης προταθεί τουλάχιστον σε ορισμένους τύπους ΣΑ. Αυτό αποδίδεται στην αλληλεπίδραση μεταξύ ιστικής και συστηματικής φλεγμονής, ανοσοκατασταλτικής θεραπείας και κοινών παραγόντων κινδύνου καρδιαγγειακής νόσου και μπορεί να συμβάλει στην αυξημένη καρδιαγγειακή νοσηρότητα και θνησιμότητα αυτών των ασθενών. Η αναγνώριση των διακριτών κλινικών φαινοτύπων ακόμη και εντός του ίδιου τύπου ΣΑ, η αναγνώριση κλινικά-σχετιζόμενων βιοδεικτών, που αφορούν τόσο τη φλεγμονώδη διεργασία όσο και τις συν-νοσηρότητες, καθώς και η μοριακή διαστρωμάτωση των ασθενών προς στοχευμένες θεραπείες εξακολουθούν να αποτελούν ανεκπλήρωτες ανάγκες.

Η παρούσα μελέτη στοχεύει να:

I. Διερευνήσει την παρουσία και την πιθανή αναστρεψιμότητα της υποκλινικής αγγειακής δυσλειτουργίας και/ή της βλάβης τόσο στη μικρο- όσο και στη μακρο-κυκλοφορία στις ΣΑ, αξιολογώντας τέσσερις κύριες αγγειακές παθολογίες (αθηρωμάτωση, αρτηριακή σκλήρυνση, αρτηριακή αναδιαμόρφωση/υπερτροφία και διαταραχή ανάκλασης κυμάτων πίεσης) σε τέσσερις διαφορετικές αγγειακές δομές (καρωτίδες, αορτή, μηριαίες αρτηρίες και αμφιβληστροειδής) χρησιμο-ποιώντας πρότυπους στην κλινική πρακτική, μη επεμβατικούς αγγειακούς βιοδείκτες. Η παρακολούθηση της υποκλινικής αγγειακής βλάβης μπορεί να παρέχει πληροφορίες για την ανάπτυξη αυτών των αγγειακών παθολογιών στις ΣΑ

καθώς και να καθοδηγήσει τη διαχείριση αυτών των ασθενών, με παρόμοιους τρόπους όπως σε άτομα με παράγοντες κινδύνου καρδιαγγειακής νόσου.

II. Εντοπίσει και να διερευνήσει τη σημασία ήδη υπαρχόντων βιοδεικτών όπως τα ANCA, καθώς και νέων συμπεριλαμβανομένου του μεταβολικού προφίλ στην παθογένεση, καθώς και για την παρακολούθηση της ενεργότητας της νόσου και της αποτελεσματικότητας της θεραπείας στις ANCA σχετιχόμενες αγγειίτιδες και την Γιγαντοκυτταρική αρτηρίτιδα (ΓΑ) αντίστοιχα.

III. Περιγράψει το κλινικό φάσμα της κρυοσφαιριναιμικής αγγειίτιδας (ΚΑ) στο πρωτοπαθές σύνδρομο Sjögren, να διερευνήσει τη σχέση του με το λέμφωμα και να εντοπίσει τις διαφορές με την ΚΑ που σχετίζεται με τον ιό της ηπατίτιδας C.

Ασθενείς και Μέθοδοι

Α. Για την αξιολόγηση της μικρο- και μακρο-κυκλοφορίας στις ΣΑ, μελετήθηκαν 73 ασθενείς με κάποιο τύπο ΣΑ, οι οποίοι αντιστοιχήθηκαν 1:1 ως προ την ηλικία, το φύλο και όλους τους παράγοντες καρδιαγγειακού κινδύνου καθώς και σχετιζόμενες θεραπείες, με υγιείς μάρτυρες και μάρτυρες νόσου ασθενείς με ρευματοειδή αρθρίτιδα (ΡΑ). Στην περίπτωση της ΓΑ, ασθενείς με ρευματική πολυμυαλγία (ΡΠ) χωρίς υποκείμενη αγγειίτιδα χρησίμευσαν ως δεύτερη ομάδα ελέγχου της νόσου. Αθηρωμάτωση (καρωτιδικές/μηριαίες πλάκες), αρτηριακή σκληρία (cfPWV), ανακλώμενα κύματα πίεσης (AIx και AIx75), αρτηριακή αναδιαμόρφωση (cIMT) και διαμέτρημα αγγείων αμφιβληστροειδούς (CRAE, CRVE) αξιολογήθηκαν τόσο σε ενεργή όσο και σε ανενεργή φάση της νόσου.

Β. Για τη διερεύνηση του μεταβολικού προφίλ σε διαδοχικούς ορούς ασθενών με ΓΑ και ΡΠ, 110 δείγματα ορού από 50 ασθενείς (33-ΓΑ και 17 ΡΠ) σε 3 χρονικά σημεία, 0-(V1: ενεργή νόσος), 1 και 6 μήνες-(V2 και V3: ύφεση) μετά από θεραπεία με γλυκοκορτικοστεροειδή, υποβλήθηκαν σε μεταβολομική ανάλυση βασισμένη σε NMR. Χρησιμοποιήθηκαν πολυ- και μονομεταβλητές στατιστικές αναλύσεις για να αποκαλύψουν μεταβολές του μεταβολισμού μετά τη θεραπεία. Επιπλέον, διερευνήσαμε την αντιγονική ειδικότητα των ANCA σε 82 P-ANCA-θετικούς ορούς με multiplex ELISA, καθώς και τη συσχέτισή τους με άλλα αυτοαντισώματα. Οι P-ANCA-θετικοί οροί αντιστοιχούσαν σε ασθενείς με ΣΑ (n=24), ΣΕΛ (n=28), (n=5), SS (n=7), ΡΑ (n=3), SSCL (n= 1), σαρκοείδωση (n=1) και θυρεοειδίτιδα Hashimoto (n= 13).

Γ. Από 1083 ασθενείς με pSS εντοπίσαμε 71 με κρυοσφαιριναιμική αγγειίτιδα. Οι ασθενείς με pSS-CV αντιστοιχίστηκαν με ασθενείς με pSS χωρίς κρυοσφαιρίνες (1:2) και ασθενείς με HCV-CV (1:1). Αναλύθηκαν τα κλινικά, εργαστηριακά χαρακτηριστικά και τα χαρακτηριστικά έκβασης. Ένα μοντέλο λογιστικής παλινδρόμησης που βασίζεται σε δεδομένα εφαρμοζόμενα για ασθενείς με pSS-CV και τους αρνητικούς για κρυοσφαιρίνες μάρτυρες pSS για τον εντοπισμό ανεξάρτητων χαρακτηριστικών που σχετίζονται με το λέμφωμα.

Αποτελέσματα

I. Η αορτική ταχύτητα σφυγμικού κύματος στις ΣΑ ήταν υψηλότερη κατά 0,7 m/sec σε σύγκριση με εκείνη των υγιών μαρτύρων και κατά 1,3 m/sec σε σχέση με ΡΑ μάρτυρες (p=0,003) και ήταν πιο έντονο στις LVV/MVV (p=0,08 και p=0,001 αντίστοιχα). Το AIx μειώθηκε σε όλες τις ΣΑ (p=0,03) και κυρίως στις SVV σε σύγκριση με τους υγιείς (p=0,04) και ΡΑ μάρτυρες (p=0,09 όλα, p=0,07 ενεργή νόσο). Ο σχηματισμός αθηρωματικής πλάκας επικράτησε σε όλες τις αγγειακές δομές κατά τη διάγνωση και ήταν πιο ενισχυμένος σε LVV/MVV (p=0,007, p=0,03, p=0,004), σε σύγκριση με υγιείς και μόνο σε καρωτίδες/μηριαίες (p=0,02) σε ΡΑ-μάρτυρες. Το πάχος έσω-μέσου χιτώνα ήταν υψηλότερο στις LVV/MVV ανεξάρτητα από την κατάσταση της νόσου και την ομάδα ελέγχου και ήταν ο πιο ευαίσθητος σε αλλαγή βιοδείκτης μεταξύ ενεργότητας και νόσου σε ύφεση. Η ενεργή ΓΑ συσχετίστηκε με αυξημένο PWV/πλάκες/cIMT σε σύγκριση με τους αντίστοιχους μάρτυρες υγιείς, ΡΑ και ΡΠ. Παρατηρήθηκε διάταση τόσο του αρτηριδίου όσο και του φλεβιδίου του αμφιβληστροειδούς σε όλες τις ενεργές ομάδες ασθενειών, ενώ η ύφεση της φλεγμονής συσχετίστηκε με μη αναστρέψιμη διάταση των αρτηριδίων σε σύγκριση με τους υγιείς (p=0,029) και ΡΑ μάρτυρες(p=0,008). Εν συντομία, διαπιστώσαμε ότι οι μη επεμβατικοί αγγειακοί βιοδείκτες επιτρέπουν την έγκαιρη αναγνώριση της αγγειακής παθολογίας κατά τη διάγνωση της νόσου, υποδηλώνοντας νόσο-ειδική και όχι σχετιζόμενη με τη θεραπεία συσχέτιση, υποδηλώνοντας έτσι ότι η έγκαιρη διαχείριση των παραγόντων κινδύνου καρδιαγγειακής νόσου, όπως προτείνεται από βιοδείκτες που αξιολογούν την υποκλινική αθηρωμάτωση και αρτηριοσκλήρωση, θα μπορούσε να αποτρέψει μακροπρόθεσμα καρδιαγγειακά συμβάντα σε ασθενείς με ΣΑ.

II. Ταυτοποιήθηκαν διαφορετικά μεταβολικά προφίλ μεταξύ ενεργότητας και ύφεσης, ανεξάρτητα από τον τύπο της νόσου. Οι N-ακετυλογλυκοπρωτεΐνες και οι χολίνες των δεσμευμένων φωσφολιπιδίων, εμφανίστηκαν ως προγνωστικοί δείκτες της δραστηριότητας της νόσου. Παρατηρήθηκαν επίσης τροποποιημένα επίπεδα 4 από τα 21 μικρά μόρια, συμπεριλαμβανομένων αυξημένων επιπέδων φαινυλαλανίνης και μειωμένης γλουταμίνης, αλανίνης και κρεατινίνης στην ενεργό νόσο. Το μεταβολικό δακτυλικό αποτύπωμα διέκρινε την ΓΑ από την ΡΠ μόνο στην ύφεση. Οι ασθενείς με ΓΑ και ΡΠ εμφάνισαν χαρακτηριστικές μεταβολές των λιπιδίων ως απόκριση και/ή ανεπιθύμητη επίδραση της θεραπείας με κορτικοστεροειδή. Η ανάλυση συσχέτισης έδειξε ότι αρκετοί ταυτοποιημένοι βιοδείκτες συσχετίστηκαν περαιτέρω με πρωτεΐνες οξείας φάσης, την C-αντιδρώσα πρωτεΐνη και την ταχύτητα καθίζησης ερυθρών αιμοσφαιρίων. Το προφίλ NMR του μεταβολισμού του ορού θα μπορούσε να αναγνωρίσει και να προτείνει ευαίσθητους βιοδείκτες φλεγμονής. Οι μεταβολές του μεταβολισμού, μετά τη θεραπεία με κορτικοστεριεδή, θα μπορούσαν να έχουν προγνωστικό ρόλο για μελλοντικές παρενέργειες που προκαλούνται από στεροειδή.

III. Στους περισσότερους θετικούς σε P-ANCA ασθενείς που μελετήθηκαν (51/82, 62,3%), αυτά τα αυτοαντισώματα εμφανίστηκαν σε υψηλούς τίτλους (>1:160). Η ανάλυση των P-ANCA-θετικών ορών αποκάλυψε αντιδραστικότητα στο MPO μόνο στο 50% των ασθενών με αγγειίτιδα, ενώ ήταν σπάνια στις άλλες ομάδες ασθενειών που μελετήθηκαν. Η αντιδραστικότητα σε άλλα αυτοαντιγόνα που σχετίζονται με το P-ANCA ανιχνεύθηκε επίσης σπάνια. Τα ευρήματά μας υποστηρίζουν ότι εμφανίζονται υψηλοί τίτλοι P-ANCA σε πλήθος συστηματικών αυτοάνοσων νοσημάτων. Το μοτίβο χρώσης με θετική P-ANCA σχετίζεται με την ειδικότητα MPO στις αγγειίτιδες και πρωτίστως στην μικροσκοπική πολυαγγειίτιδα, ενώ σε άλλες αυτοάνοσες ασθένειες, ως επί το πλείστον περιλαμβάνει άγνωστα αυτοαντιγόνα.

IV. Οι ασθενείς με pSS-CV είχαν υψηλότερη συχνότητα εξωαδενικών εκδηλώσεων και λεμφώματος (OR=9,87, 95% CI: 4,7-20,9) σε σύγκριση με ασθενείς με pSS χωρίς κρυοσφαιρίνες. Η πορφύρα ήταν η συχνότερη αγγειακή εκδήλωση (90%), με εμφάνιση κατά την έναρξη της νόσου στο 39% των ασθενών. Το ένα τρίτο των ασθενών με pSS-CV ανέπτυξαν λέμφωμα Β-κυττάρων μέσα στα πρώτα 5 χρόνια της πορείας της κρυσφαιριναιμικής αγγειίτιδας, με την κρυοσφαιριναιμία να είναι ο

ισχυρότερος παράγοντας που σχετίστηκε με ανάπτυξη λεμφώματος. Σε σύγκριση με τους ασθενείς με HCV-CV, τα άτομα με pSS-CV εμφάνισαν συχνότερα λεμφαδενοπάθεια, κρυοσφαιρίνες IgMk τύπου II και λέμφωμα (OR = 6,12, 95% CI: 2,7-14,4) και σπανιότερα C4 υποσυμπληρωμαιμία και περιφερική νευροπάθεια. Συμπερασματικά, το η κρυοσφαιριναιμική αγγειίτιδα στο πλαίσιο SS έχει σοβαρή κλινική πορεία, επισκιάζοντας τις τυπικές κλινικές εκδηλώσεις του SS και υψηλότερο κίνδυνο για πρώιμη ανάπτυξη λεμφώματος σε σύγκριση με την κρυοσφαιριναιμική αγγειίτιδα που σχετίζεται με τον HCV. Αν και σπάνια, η κρυοσφαιριναιμική αγγειίτιδα αποτελεί έναν ξεχωριστό σοβαρό κλινικό φαινότυπο του SS.

Μελλοντικές μελέτες με μακροχρόνια παρακολούθηση και διαστρωμάτωση σύμφωνα με την ανοσοκατασταλτική θεραπεία θα διευκολύνουν την καλύτερη κατανόηση της σχέσης μεταξύ αγγειακής παθολογίας και φλεγμονής, παρέχοντας ωστόσο νέους βιοδείκτες για τη διάγνωση, την πρόληψη και την απόκριση στη θεραπεία.

Publications from the present thesis

1. Argyropoulou OD, Argyris AA, Mouziouras D, AissopouEK, Charalampopoulos G, Mavragani CP, Tentolouris N, Sfikakis PP, Anagnostopoulos CD, Vlachoyiannopoulos PG, Protogerou AD. Non-invasive vascular biomarkers identify early vascular damage and guide the management of cardiovascular risk factors in Primary Systemic Vasculitis. Manuscript submitted.
2. Iliou*A, Argyropoulou OD*, Palamidas DA, Karagiannakou M, Benaki D, Tsezou K, Vlachoyiannopoulos PG, Mikros E**,Tzioufas AG**. NMR-based Metabolomics in Giant Cell Arteritis and Polymyalgia Rheumatica sequential sera differentiates active and inactive disease. In press, Rheumatology (Oxford).*, ** shared first and last name respectively.
3. Argyropoulou OD, Goules AV, Boutzios G, Tsirogianni A, Sfontouris C, Manousakis MN, Vlachoyiannopoulos PG, Tzioufas AG, Kapsogeorgou EK. Occurrence and Antigenic Specificity of Perinuclear Anti-Neutrophil Cytoplasmic Antibodies (P-ANCA) in Systemic Autoimmune Diseases. Cells. 2021 Aug 19;10(8):2128.
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The full-text version of the above-mentioned articles is available in *Appendix*.

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Appendix

REVIEW

Accelerated atheromatosis and arteriosclerosis in primary systemic vasculitides: current evidence and future perspectives

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Purpose of review
Primary systemic vasculitides (PSV) encompass a subset of autoimmune diseases, characterized by inflammation of blood vessels. Atheromatosis and arteriosclerosis may be accelerated in several PSV and account for the increased rate of cardiovascular morbidity that some exhibit. We aimed to summarize recent studies reporting on the acceleration of atheromatosis and/or arteriosclerosis in each type of PSV, using state-of-the-art noninvasive vascular biomarkers with clinical value as end points.

Recent findings
Limited number of PSV patients and methodology limitations reduce the value of many published studies. Accelerated atheromatosis, as measured by the use of carotid ultrasonagraphy (plaques and intimal–medial thickening) and increased arterial stiffening, as measured by the use of applanation tonometry (carotid to femoral pulse wave velocity), are currenly well established in Takayasu arteritis, Kawasaki disease and Behcet's disease. The association of atheromatosis and arteriosclerosis with polyarteritis nodosa and small vessel vasculitides remains less established and studied, so far.

Summary
Accelerated atheromatosis and arteriosclerosis or arteriosclerosis are established in some PSV. The potential clinical value of easy-to-measure and clinically useful noninvasive vascular biomarkes prompts the need for large prospective cohorts in order to provide useful future guidance regarding the prognosis and treatment of PSV patients.

Keywords
arteriosclerosis, atheromatosis, intima–media thickness, primary vasculitides, pulse wave velocity

INTRODUCTION

Primary systemic vasculitides (PSV) is a heterogeneous group of rare and potentially life-threatening diseases characterized by inflammation of the vascular wall [1▪,2]. The size and localization [4] of the involved vessels in association with the nature of the inflammatory process (focal or systemic, presence of necrosis, immune complex formation) account for the variability of the clinical manifestations between the various PSV [2]. Prior to the introduction of corticosteroids, the natural history of untreated PSV was that of a rapidly progressive and usually fatal disease [3,4]. Nowadays, the causes of death include cancer and infections because of chronic immune activation and/or immunosuppressive therapy [4,6]. Premature deaths may also occur because of acute renal failure and pulmonary hemorrhage, especially in small vessel vasculitides [5], whereas macrovascular complications (e.g. coronary artery disease, stroke, aneurysm formation and rupture) are the leading causes in medium and large vessel vasculitides [3,4,5]. Vascular damage in PSV is primarily characterized by lumen stenosis, occlusion or aneurysmal dilatation of blood vessels because of intramural inflammation and necrosis [3]. Mural fibrin deposition in arterioles or venules as well as angiocentric inflammatory cell infiltration are the hallmarks of biopsy-proven

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KEY POINTS

- The limited number of primary systemic vasculitides (PSV) patients and methodology limitations (lack of prospective data, often misuse the interpretation of vascular biomarkers) reduces the value of many published studies.
- Strong data on the acceleration of atheromatosis and increased arterial stiffening are currently present in Takayasu arteritis, Kawasaki disease and Behcet's disease.
- The association of atheromatosis and arteriosclerosis with polyarteritis nodosa and small vessel vasculitides remains the least established, so far.
- The potential clinical value of noninvasive vascular biomarkers such as carotid intimal–medial thickening and carotid to femoral pulse wave velocity prompts the need for large prospective cohorts in order to provide useful future guidance regarding the prognosis and treatment of PSV patients.

diagnosis [3]. The early steps in the immunological process of vascular damage in PSV cannot be considered to be uniform, as various discrete mechanisms such as endothelial activation and dysfunction, autoantibodies to endothelial cell-surface antigens or neutrophil components and abnormal IgA tissue deposition are involved [5]. Moreover, emerging evidence suggest that the two classical pathways of arterial damage, namely, atheromatosis (i.e. atheromatic plaque formation), and arteriosclerosis (i.e. arterial stiffening), are accelerated, thus participating in the development of microvascular and macrovascular complications in PSV [4,7▪▪,8]. Herein, we aim to summarize recent studies reporting on accelerated atheromatosis and arteriosclerosis or arteriosclerosis in each type of PSV. To this end, we also present the major, widely applied noninvasive vascular biomarkers used to assess atheromatosis [intima–media thickness (IMT), plaque presence] and arteriosclerosis [pulse wave velocity (PWV) and carotid disentibility] in clinical research and practice. Secondary vasculitides have been excluded from the present review, as these entities involve additional pathological mechanisms.

POTENTIAL MECHANISMS OF ACCELERATED ATHEROMATOSIS AND ARTERIOSCLEROSIS IN PRIMARY SYSTEMIC VASCULITIDES

Although it is widely accepted that atherosclerosis involves an ongoing inflammatory response [9], the potential mechanisms of this phenomenon are yet poorly studied in PSV. Although major differences do exist, one might consider that the acceleration of arterial damage in PSV shares several common mechanisms with rheumatoid arthritis (RA), which is by far, the most extensively studied model of arterial damage in chronic inflammatory diseases [10,11]. Therefore, cautious extrapolation of these mechanisms to PSV seems reasonable. In brief, these mechanisms involve: the primary intramural vessel wall inflammation (Box 1, Fig. 1); the secondary vessel wall inflammation by systemic inflammation (Box 2. Figure 1); PSV-related drug treatment-induced deleterious effects on the vessel wall

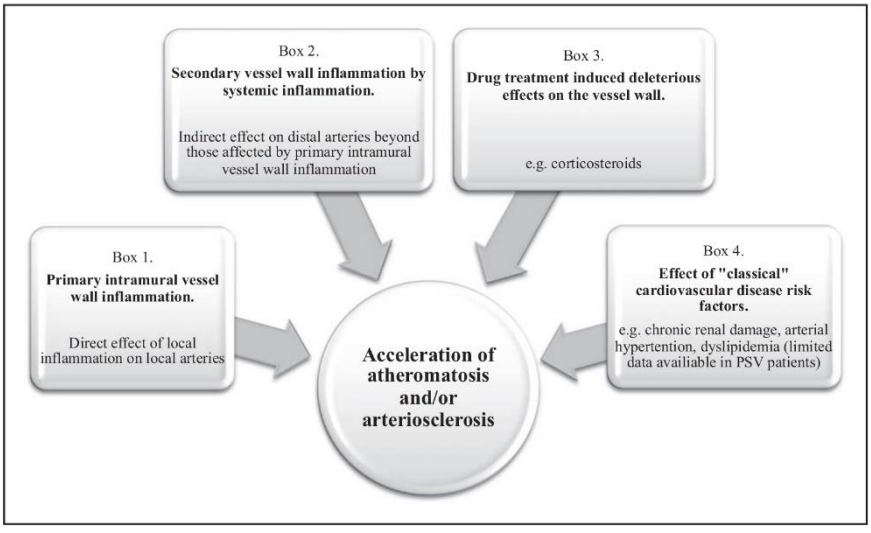

FIGURE 1. Potential mechanisms leading to the acceleration of atheromatosis and arteriosclerosis in primary systemic vasculitides (PSV).

(Box 3, Fig. 1) and the effect of classical cardiovascular disease risk factors (Box 4, Fig. 1) [7▪▪].

Data on the prevalence of hypertension and dyslipidemia are currently lacking in PSV patients, but an increased incidence compared with the general population is thought to result from the chronic use of corticosteroids, the often presence of chronic renal damage, as well as the chronic inflammatory process per se. These general mechanisms may not only precipitate the classical inflammatory process of atheromatosis [7▪▪], but also may account for the disruption of the balance between synthesis and degradation of collagen and elastin, leading to vascular stiffening [8].

NON-INVASIVE VASCULAR BIOMARKERS FOR THE ASSESSMENT OF ATHEROMATOSIS AND ARTERIOSCLEROSIS

Atheromatosis (atheromatic plaque formation) and arteriosclerosis (reduced elasity because of elastin fiber loss or dysfunction) represent two distinct pathways of arterial damage, which although share some common risk factors but have different damage and pathophysiological consequences. A large variety of noninvasively assessed vascular biomarkers have been developed during the past 30 years to describe these two pathways [12▪▪]. The most widely applied and herein used biomarkers are described in Table 1. These biomarkers are used to investigate the damage of the artery and optimize cardiovascular risk stratification in clinical practice but currently cannot be used in treatment follow-up [12▪▪].

LARGE VESSEL VASCULITIDES

Takayasu arteritis

Takayasu arteritis is a chronic inflammatory granulomatous vasculitis, manifesting mainly as a panaortitis and occuring commonly in young women between 10 and 30 years [13]. Takayasu arteritis is associated with a significantly increased risk of cardiovascular complications, including cerebrovascular events, aortic aneurysm formation and ruptured and congestive heart failure. The mechanisms that link Takayasu arteritis with late cardiovascular complications remain to be fully elucidated [15].

In Takayasu arteritis, the inflammatory process commences from the adventitia and progresses to the intima leading to segmental stenosis, occlusion, dilatation and aneurysm or aneurysm formation. Histologically, it is characterized as a 'panarteritis' involving all layers of the arterial wall, including intimal fibrous thickening and/or typical atheromatous lesions, destruction of medial smooth muscles and elastic layers, cellular infiltration and collagenous fibrosis in the media and thickened adventia with cellular infiltration around vasa vasorum. Intact areas between affected areas in arteries ('skipped lesions') are usually revealed in pathological studies [14].

Although the number of Takayasu arteritis patients is still limited, Takayasu arteritis is the most well studied PSV regarding the mechanisms of atheromatosis and arteriosclerosis. Most studies have demonstrated that carotid artery IMT (cIMT) is significantly higher in the Takayasu arteritis group as compared with the control group [16▪▪,19,20▪,21,22]. Moreover, high prevalence of atheromatic plaques is seen and can not be explained by the traditional vascular risk factors [16▪▪,18▪,19,20▪,21]. Of note, it was suggested that the abnormal cIMT might be used as a reliable marker of disease activity in Takayasu arteritis (sensitivity of 82% and specificity of 60%) and that it should be part of the routine evaluation of Takayasu arteritis [21,22]. In daily practice, clinicians may have difficulty in making differential diagnosis between Takayasu arteritis-related vascular lesions

Table 1. Noninvasive vascular biomarkers used for the study of atheromatosis and arteriosclerosis

	Intima–media thickness (IMT)	Plaque presence	Carotid–femoral pulse wave velocity (cfPMV)
Measured by	Ultrasonography	Ultrasonography	Applanation tonometry (most widely applied)
Disease studied	Arterial remodeling and/or atheromatosis (at the level of the common carotid artery)	Atheromatosis	Arteriosclerosis (arterial stiffening)
Arterial bed studied	Carotid	Carotid and femoral	Aorta (thoracic and abdominal)
Recommendation to be used in clinical practice in population at intermediate cardiovascular disease risk populations	Yes	Yes	Yes

and atheromatosis. Ugurlu *et al.* in a study of 58 patients investigated the morphologic and hemodynamic changes in the carotid arteries in Takayasu arteritis, along with patients with diabetes mellitus and healthy controls using Doppler ultrasonography (USG). The study showed that carotid artery may be helpful in differentiating Takayasu arteritis from atherosclerosis. Diffuse homogenous increase in IMT, presence of turbulence and higher resistivity index can be considered as suggestive of Takayasu arteritis rather than atherosclerosis [17▪▪].

Increasing data also suggest that Takayasu arteritis is associated with elevated arterial stiffness in the central elastic arteries compared with controls and that arterial stiffness may persist even when the disease is quiescent. PWV was found to be significantly increased in Takayasu arteritis patients, despite the younger age and the comparable blood pressure with the control group implying that structural rather than functional vascular damage takes place in Takayasu arteritis [15,23].

Giant cell arteritis

Giant cell arteritis (GCA), the most common granulomatous PSV with a predilection for large-sized and medium-sized arteries, occurs almost exclusively after the age of 50 years and affects mainly the extracranial branches of the carotid artery. It is associated with doubled incidence of cardiovascular events and 17 times increased risk for aortic aneurysms [24▪,25,26,31].

Similar to Takayasu arteritis, GCA inflammation involves all layers of the arterial wall and the inflammatory process appears to begin in the adventitial layer at the level of vasa vasorum. Granulomatous infiltrate including giant cells is proposed to play a key role in the invasion from the adventitial side to the medial and intimal layers [28].

Several early case reports (including few patients) describe a vasculitic carotid wall thickening in GCA [28], but there are also data showing significantly lower cIMT levels compared with controls [27]. A study of 41 GCA patients showed that steroid therapy has no influence on endothelial function but does significantly improves cIMT in GCA. Increasing data demonstrate that IMT measurement of temporal, facial and axillary arteries can correctly distinguish vasculitic from normal arteries in suspected GCA, thus IMT cut-off values may additionally help in the diagnosis of GCA [29▪]. Data for wall diameters are needed for future longitudinal trials to monitor GCA treatment.

Limited data are availiable concerning the potential effect of GCA on arterial stiffness. A study of 49 patients showed that GCA patients have higher PWV and dilated thoracic aortas with a women preponderance compared with GCA men. Further investigation is required to evaluate the effect of severity, treatment length, disease duration and cardiovascular risk factors on aortic morphology and function [30▪].

MEDIUM VESSEL VASCULITIDES

Polyarteritis nodosa

Polyarteritis nodosa (PAN) is an extremely rare, necrotizing vasculitis associated with aneurysmal nodules along the walls of medium-sized muscular arteries that can present initially as peripheral vascular ischemia [32,33]. Poor data, involving only a few patients have been found in the literature leading to inconclusive results for the development of accelerated atheromatosis [2,33,34,35,36].

Kawasaki disease

Kawasaki disease ia an acute medium vessel vasculitis, occuring predominantly in infants and during early childhood. The most significant complication is the development of coronary aneurysms during the subacute phase. These aneurysms are known to cause coronary artery disease by causing thrombosis and stenosis and represent a cause of sudden death in this patient group [37,38]. A review of autopsies from Kawasaki disease patients revealed that the arterial damage includes necrotizing arteritis, subacute or chronic vasculitis and luminal myofibroblastic proliferation [42▪].

Recent literature implicates in this, various factors like endothelial dysfunction, proatherogenic lipid profiles and arterial stiffening [38]. It is unclear whether all children with Kawasaki disease have increased later cardiovascular risk. The retinal microvasculature reflects changes in the microcirculation and is associated with traditional cardiovascular risk factors and events. Larger retinal venules may reflect chronic inflammation and endothelial dysfunction, and are associated with coronary artery disease in adults [39▪▪].

Carotid artery IMT is one of the most commonly used noninvasive measures of subclinical atherosclerosis in both pediatric and adult populations. There are few studies showing higher cIMT in children with Kawasaki disease compared with controls and others that demonstrate higher cIMT in patients of Kawasaki disease with coronary aneurysms [38]. As cIMT and aortic IMT have been shown to be a surrogate marker of both coronary and peritheral atherosclerosis, higher cIMT and aortic IMT in children with Kawasaki disease along with

proatherogenic abnormatiries in lipid profile may predict a higher risk of coronary artery events in later life [38,40▪▪,41▪▪,43▪].

Numerous studies have demonstrated the association between Kawasaki disease and arteriosclerosis. Overall patients with a history of Kawasaki disease exhibited a high PWV relative to controls. This suggests that these patients have a subsequent tendency for increased arterial stiffness. Consequently, life-long follow-up should be advised to evaluate cardiovascular diseases caused by former Kawasaki disease vasculitis and age-associated factors [37,44,45▪,46▪].

SMALL VESSEL VASCULITIDES

Antineutrophil cytoplasmic antibody-associated vasculitides

Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitides (AAV) include three clinical entities: granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPO), eosinophilic GPA (EGPA or Churg–Strauss syndrome). They are necrotizing vasculitis with few or no immune deposits affecting predominantly small vessels [1▪,4]. GPA and EGPA are also characterized by granulomatous inflammation [4]. Although AAV share several clinical and histopathological features, they represent three distinct diseases, mainly on the basis of ANCA antigen specificity [1▪].

Compared with the general population, the risk of cardiovascular disease (CVD; including MI, stroke and aneurysm formation) is two-fold to four-fold higher among patients with AAV, thus exhibiting enhanced cardiovascular mortality [3,7▪▪]. Patients with PR3 ANCA have a lower CVD risk than those with MPO ANCAs [47]. This is based on the fact that myeloperoxidase (a granule protein identified inside human atherosclerotic lesions and expressed in leucocytes) [49] has been implicated both in the initiation and progression of atheromatosis [7▪▪,48].

Several studies, most of which concern GPA [20▪,50,51▪] have shown increased common carotid IMT (ccIMT) in AAV patients as compared with controls [2,52], thus suggesting that AAV are associated with accelerated and frequently subclinical atheromatosis that can not be explained by traditional risk factors. Furthermore, the raised levels of high sensitivity C-reactive protein, MMPs and tissue inhibitor of metalloproteinase (TIMP) suggest that enhanced inflammation and excessive vascular remodelling are contributing factors in the development of accelerated atheromatosis in GPA [50]. González-Suárez *et al.* [53▪] in a study of 23 patients, observed an association between carotid intima–media thickness and internal carotid artery pulsatility index with small vessel cerebral disease pointing the possible use of carotid ultrasonography in predicting microvascular brain injury. Studies with a good number of MPO and EGPA patients were not found.

Only few data are availiable concerning the arterial stiffness as assessed by PWV measurement and the atherogenic index in AAV. Two studies with limited number of patients have shown increased arterial stiffness, and that arterial stiffness correlates with the degree of active inflammation in AAV patients [54,55].

As far as other small vessel vasculitides (i.e. purpura henoch schonlein and cryoglobulinemia vasculitis) are concerned, no reliable data showing accelerated atheromatosis and arteriosclerosis were found.

BEHCET'S DISEASE

Behcet's disease is a chronic, relapsing, multisystemic vasculitis involving both veins and arteries of any size [56]. In adition to the classic triad (recurrent apthous ulcers, genital ulcers, uveitis), pulmonary, gastrointestinal, nervous and musculoskeletal manifestations may be present [58▪▪]. Cardiovascular involvement (deep vein thrombosis, MI, arterial aneurysm, arterial thrombus formation) [59], occurs in 7–31% of patients and is associated with poor prognosis and increased mortality in patients with major vessel involvement [56].

Histopathologically, Behcet's disease is mainly characterized by vasculitis, with prominent neutrophil and monocyte infiltration in the perivascular regions with or without fibrin deposition in the vessel wall. Endothelial dysfunction, the initial lesion in atheromatosis, as well as the intermittent inflammation, autoimmune mechanisms and drugs are thought to account for the accelerated atherosclerosis in patients with Behcet's disease [58▪▪]. Of note, endothelial dysfunction in patients with Behcet's disease is modulated by the presence of corticosteroids and disease activity status. During disease relapse, corticosteroids restore endothelial dysfunction but their prolonged administration in the absence of active disease may be detrimental for the endothelium [57].

Despite previous evidence demonstrating atherosclerosis as not a prominent feature of Behcet's disease, even among patients with major organ involvement [60], a recent meta-analysis of relevant studies [61▪▪] showed that cIMT is increased in patients with Behcet's disease compared with controls. Similarly, the same meta-analysis showed that carotid plaques are three times more prevelent in

Table 2. Summary of data regarding the presence of accelerated atheromatosis and arteriosclerosis in primary systemic vasculitides

	Evidence of increased incidence of cardiovascular events	Evidence of accelerated atherosclerosis	Evidence of accelerated arteriosclerosis
Takayasu arteritis	++ [13,15]	++ [16▪▪,19,20▪,21,22]	+ [15,23]
Giant cell arteritis	++ [24▪,25,26,31]	++ [27,28,29▪]	+ [30▪]
Polyarteritis nodosa	+ [32,33]	□	□
Kawasaki disease	++ [37,38]	++ [38,40▪▪,41▪▪,42▪,43▪]	++ [37,44,45▪,46▪]
ANCA-associated vasculitides[a]	++ [3,7▪▪]	+ + [2,20▪,50,51▪,52,53▪]	+ [54,55]
Henoch Schonlein	□	□	□
Cryoglobulinemia vasculitis	□	□	□
Behcet's disease	++ [56,59]	+++ [61▪▪,62,63▪,64]	++ [58▪▪,65▪▪]

□, Lack of data; +, data derived from one or two single-center cohorts; ++, large number of evidence derived from multiple single-center cohorts; +++, evidence based on meta-analysis, ANCA, antineutrophil cytoplasmic antibodies.
[a]The evidence concerns granulomatosis with polyangiitis.

patients with Behcet's disease compared with the control group, verifying the presence of accelerated subclinical atheromatosis [58▪▪,61▪▪]. Another study including 50 patients showed that Behcet's disease may be associated with subtle increased cIMT, suggesting that it can be a predisposing factor for atherosclerotic arterial disease [62]. Further more, Cure *et al.* [63▪] in a cross-sectional case–control study, showed a strong positive correlation between atherogenic index of plasma (AIP) and cIMT. These and other results [64] suggest that accelerated subclinical atherosclerosis might explain the presence of increased cardiovascular events and mortality in these patients.

Moreover, several studies showed increased arterial stiffness, assessed by carotid to femoral PWV [58▪▪]. A study of 30 patients showed that PWV measurement might be more useful than cIMT in determination of vascular damage in Behcet's disease, especially in early stage of disease duration [65▪▪].

CONCLUSION

Despite the fact that there are no international networks dedicated to the study of cardiovascular risk factors, the role of PSV-drugs (e.g. corticosteroids) and vascular properties in PSV, recent studies using noninvasive techniques (cIMT and PWV) have demonstrated accelerated atheromatosis and increased arterial stiffening in these patients, thus suggesting a potential role for the increased cardiovascular events and associated mortality. Because of the limited number of PSV patients and the lack of prospective data and experience on vascular biomarkers by most rheumatologists, many PSV, especially PAN and small vessel vasculitides, remain under-studied (Table 2). The potential clinical value suggested by the so-far-studied vascular biomarkers would allow rheumatologists, implement optimal therapeutic strategies in the clinical practice in order to reduce the increased cardiovascular morbidity and mortality in PSV. This hypothesis prompts the need for large prospective cohorts that will record cardiovascular disease risk factors and apply the methods discussed herein, as well as other noninvasive methods, in order to provide useful future guidance regarding the evaluation and restratification of cardiovascular risk, which should lead to optimization of the prognosis and treatment of PSV patients.

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There are no conflicts of interest.

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RESEARCH PROTOCOLS-PROPOSAL

Extensive phenotyping of vascular damage in non-infectious primary vasculitides with the use of non-invasive vascular biomarkers: prevalence, pathogenesis and response to treatment

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ABSTRACT

Non-Infectious Primary systemic vasculitides (NIPSV) encompass a subset of autoimmune diseases, characterized mainly by intramural inflammation of the vascular wall. The increased mortality that some exhibit is partially attributed to vascular complications involving both micro- and macro- circulation. Beyond the disease specific pathways of vascular damage, emerging evidence suggest that the classical pathways of arterial damage, namely, atheromatosis, inappropriate arterial remodeling and arteriosclerosis are accelerated in several NIPSV; thus participating in the development of vascular complications in NIPSV patients. The aim of the current research protocol is to optimize the understanding of vascular pathology in NIPSV and to identify useful, easy to measure, non-invasive vascular tools for the diagnosis and follow-up of NIPSV patients. Moreover, the study aims to generate hypothesis regarding the molecular basis of the association of inflammation with classical vascular pathology.

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INTRODUCTION

Non-Infectious Primary Systemic Vasculitides (NIPSV) is a heterogeneous group of rare and potentially life-threatening diseases characterized by inflammation of the vascular wall.[1,2] The size and localization of the involved vessels in association with the nature of the inflammatory process (focal or systemic, presence of necrosis, immune complex formation) account for the variability of the clinical manifestations between the various NIPSV.[2] Prior to the introduction of corticosteroids, the natural history of untreated NIPSV was that of a rapidly progressive and

usually fatal disease.[3,4] Nowadays, the causes of death include cancer and infections due to chronic immune activation and/or to immunosuppressive therapy.[4,6] Vascular complications (involving both micro- and macro-circulation) are major sources of morbidity and mortality in NIPSV. Microvascular complications are major causes of premature deaths especially in small vessel vasculitides: they affect predominantly the kidney (acute renal failure) and the lung (pulmonary hemorrhage).[5] Macrovascular complications (e.g., coronary artery disease, stroke, aneurysm formation and rupture) are major causes of morbidity and mortality especially in medium and large vessel vasculitides.[3,4,5] Lumen stenosis, occlusion or aneurysmal dilatation of blood vessels due to intramural inflammation and necrosis represent the major vascular pathology in NIPSV.[3] Mural fibrin deposition in arterioles or venules, as well as angiocentric inflammatory cell infiltration are the hallmarks of biopsy-proven diagnosis.[3] The early steps in the immunological process of vascular damage in NIPSV cannot be considered to be uniform, since various discrete mechanisms such as endothelial activation and dysfunction, autoantibodies to endothelial cell-surface antigens or neutrophil components and abnormal IgA tissue deposition are involved.[5]

Beyond the NIPSV specific pathways of vascular damage, numerous evidence suggests that the classical pathways of arterial damage, namely, atheromatosis (i.e., atheromatic plaque formation), inappropriate arterial remodeling (e.g., arterial hypertrophy / distention) and arteriosclerosis (i.e., arterial stiffening), are accelerated, thus participating in the development of micro- and macro-vascular complications in NIPSV.[4,7,8] In brief, the main related mechanisms involve: (i) the primary intramural vessel wall inflammation; (ii) the secondary vessel wall inflammation due to systemic inflammation; (iii) NIPSV–related drug treatment induced deleterious effects on the vessel wall; and (iv) the effect of classical cardiovascular disease risk factors.[7]

However, numerous unanswered questions exist due to the limited number of NIPSV patients and methodological limitations (lack of prospective data, often misused interpretation of vascular biomarkers) that reduce the value of many published studies. Strong data on the acceleration of vascular damage (atheromatosis and increased arterial stiffening) are currently present in Takayasu Arteritis, Kawasaki Disease and Behcet's Disease.[9] The association of atheromatosis and arteriosclerosis with PN and small vessel vasculitides remains the less established, so far.[9] The actual contribution of classical vascular pathology in the development of morbidity and mortality in NIPSV is not known. The effect of anti-inflammatory drugs on the function and structure of the large and small arteries has been scarcely evaluated in NIPSV. The potential clinical value of non-invasive vascular biomarkers such as carotid intimal-medial thickening and carotid-to-femoral pulse wave velocity prompts the need for large prospective cohorts in order to provide useful future guidance regarding the prognosis and treatment of PSV patients.[9]

AIMS OF THE STUDY

Primary aims: (i) to identify the frequency of vascular damage, studied per pathology (atheromatosis, arteriosclerosis, remodeling) in the overall population, as well as, per disease, and compare it to the corresponding frequency of an age- and gender-matched control group; (ii) to identify vascular phenotypes, i.e., patterns of vascular damage (micro- and macro- circulation) per disease as well per vascular bed within each disease, as well as differences between the diseases; (iii) to quantify the relative contribution of each one of the above proposed mechanisms in the development of vascular damage in NIPSV; (iv) to identify vascular biomarkers (e.g., as the previously proposed "augmentation index") that associate with disease activity in NIPSV; and (v) to study the effect of anti-inflammatory treatment from disease onset (or relapse) to complete remission and follow-up. To test the previously described hypothesis of biphasic effect of corticosteroids on the function and structure of the large and small arteries.

Secondary aims: (i) to identify the prevalence of classical cardiovascular diseases risk factors (hypertension, dyslipidemia, smoking, diabetes) in NIPSV, using state-of-the-art methodology (e.g., out-of-office blood pressure monitoring); (ii) Explorative & hypothesis generating analysis: DNA and miRNA will be extracted to stored. NIPSV considered as prototype of high grade vascular wall inflammation with extreme flares and periods of complete remission, will be used as a model to perform exploratory analysis leading to novel hypothesis regarding the molecular pathogenesis vascular disease linked to inflammation. Future perspectives (in a *separate 2nd phase study):* In a future follow-up study of the present cohort, we will evaluate the prognostic value of these biomarkers to predict morbidity and mortality and to be used as valid biomarkers to guide diagnosis and response to treatment.

METHODS

Prospective observational study; anticipated study duration (based on 6 month pilot study is calculated around 3 years (2018-2021); sample size: 200 (25 per disease group at least) patients and 400 age- and gender-matched individuals without chronic inflammatory disease, NSIP or history of neoplastic disease who will serve as controls. Baseline and follow-up vascular evaluation of all the participants will be performed in pre-defined visits on the basis of disease activity, remission and relapse, as defined by international guidelines per disease (**Table 1**). Patients with any type of NIPSV - diagnosed on the basis of classical international criteria per disease - fulfilling the inclusion criteria will be recruited in one of the following 3

Table 1: Study flow chart

Visit	Hospitalized	Duration	Anticipated disease status	NIPSV-Related treatment		Cardiovascular treatment	Vascular tests	PET/ CT*	Blood tests**	Biopsies
				Cortico-steroids	Immuno-suppressive treatment					
0	yes	0	Active Disease (newly diagnosed or disease relapse)	Naive or steady for months	Naive or steady	Steady if possible	x	x	x	x If possible
1	yes	<7 days	Still active disease (newly diagnosed or relapse)	1-2 just after IV high dose completion therapy	1-2 days after 1st dose	Steady if possible	x	+/-	x	+/-
2	no	1st month	«Start» of remission	Just before dose tapering	Steady if possible	Steady if possible	x		x	
3	no	3rd month	Remission	Tapering completion (start of minimal dose or no dose)	Steady if possible	Steady if possible	x		x	
4	no	6th month	Remission	Steady	Steady if possible	Steady if possible	x		x	

Legend

- Visit 0 for newly diagnosed patient (group A); visit 1 for disease relapse (group B); visit 3 for patient in steady disease remission status for at least 3 months with steady drugs (group C).
- * PET/ CT will be performed at the Biomedical Research Foundation Academy of Athens (BRFAA) only for visits 0 or 1 and to verify complete disease remission (visit 4).
- ** Renal function, metabolic profile, inflammation profile and disease specific test if need will be performed.

Serum from all visits will be stored anonymous at the biobank of cells and tissues of the Department of Pathophysiology of Medical School of National & Kapodistrian University of Athens.

groups: Group A: consecutive newly diagnosed patient with NIPSV; Group B: NIPSV with active relapse; Group C: consecutive NIPSV patients in steady disease - remission status for at least 3 months with steady medication. Age- and gender-matched individuals without any chronic inflammatory disease and NIPSV will serve as control group. Extensive vascular studies with high resolution ultrasound, oscillometry and tonometry will be performed at the carotid bed, the femoral bed, the aorta, the upper arm and lower limbs and retina in order to evaluate atheromatosis, arteriosclerosis/elasticity, arterial remodeling and hypertrophy endothelial function, wave reflections and aortic hemodynamics, retinal geometry (**Table 2**) in 2 to 5 sequential visits within 6-9 months (visit 0, visit 1=1 week, visit 2= 1 month, visit 3= 3 months, visit 4 ≥ 6 months) in each patient starting from disease onset/ relapse to complete remission. Anthropometric parameters, dietary habits, lipids and other blood samples, DNA, RNA, urine samples, tissues biopsies, PET/CT will be recorded in predefined visits.

Patients involved in the study are informed in detail and give written consent. All data are collected under code in anonymous electronic files, in which only researchers have access, at the Cardiovascular Prevention & Research Unit of the Department of Pathophysiology of Medical School of National & Kapodistrian University of Athens. Under the above particular circumstances, biological materials; blood serum, DNA, RNA and biopsy tissues are collected and retained anonymous at the biobank of cells and tissues of the Department of Pathophysiology of Medical School of National & Kapodistrian University of Athens. Ethical approval has been obtained

Table 2. Assessment of macro- and micro-circulation.

Macrocirculation - *vascular bed, vascular-hemodynamic properties and non-invasive vascular biomarkers that will be assessed* • Carotid to femoral pulse wave velocity (cfPWV) by pulse wave analysis (PWA (elastic arteries arteriosclerosis); Sphygomocr device • Carotid to radial PWV by PWA (muscular arteries arteriosclerosis); high resolution ultrasound; GE Logic V5 • Carotid elasticity (elastic arteries arteriosclerosis) (right and left); high resolution ultrasound; GE Logic V5 • Carotid artery wall to lumen ratio (arterial remodelling) (right and left); high resolution ultrasound; GE Logic V5 • Carotid artery intimal-medial thickness (IMT) (right and left)(arterial hypertrophy / atheromatosis); high resolution ultrasound; GE Logic V5 • Carotid (common, bulb, internal; right and left) bed plaques (atheromatosis); high resolution ultrasound; GE Logic V5 • Femoral bed - plaques (atheromatosis); high resolution ultrasound; GE Logic V5 • Ankle-brachial index (atheromatosis and arteriosclerosis); oscillometric device; Micrlife office BP - ABI. • Brachial artery flow-mediated dilatation (endothealial function); high resolution ultrasound; GE Logic V5 • Abdominal aorta and subclavian artery diameters; high resolution ultrasound; GE Logic V5 • Aortic blood pressure, subendocardial viability index by PWA; Sphygomocr device • Twenty-four hours ambulatory aortic stiffness, aortic blood pressure, brachial blood pressure, cardiac output and total prepheral resistance monitoring; Mobilograph IEM device •
Microcirculation - vascular bed, vascular-hemodynamic properties and non-invasive vascular biomarkers that will be assessed • Retinal microcirculation by digital camera photography (and dedicated software analysis (Imedos) • Pressure wave reflections (augmentation index) by PWA; Sphygomocr device • Pressure wave reflections by twenty-four hourw ambulatory aortic hemodynamic monitoring; Mobilograph IEM device

by the Bioethics and Ethics Committee of National and Kapodistrian University of Athens.

Statistical analysis

Statistical analysis will be performed by SPSS v. 23.0 using appropriate test per hypothesis and populations. The data will be analyzed and presented in the overall population as well per disease and disease status, after normal distribution control. Comparison of the outcome variables (vascular indices) between and within groups will be performed before and after adjusting for potential confounders using independent multiple t-tests, ANOVA, paired t-test, linear and logistic regression analysis, and chi-square tests as appropriate. Receiver operator curve analysis will be performed to identify ability of the outcome variables to detect disease activity. Sensitivity and mediation analysis will be performed.

ANTICIPATED BENEFITS:

- To optimize the understanding of vascular pathology in NIPSV.
- To identify potential useful easy to measure non-invasive vascular tools for diagnosis and follow-up of NIPSV patients.

- To generate hypothesis regarding the molecular basis of association of inflammation with classical vascular pathology (atheromatosis, arteriolosclerosis, arterial remodeling).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Common and rare forms of vasculitis associated with Sjögren's syndrome

Ourania D. Argyropoulou and Athanasios G. Tzioufas

Purpose of review
Although uncommon, systemic vasculitis is one of the most severe extraglandular manifestations of primary Sjögren's syndrome (pSS) accounting for the increased morbidity and mortality of the disease. This review aims to describe major previous and recent reports regarding the clinical presentation, prognosis and treatment of systemic vasculitis associated with pSS.

Recent findings
Both older and recent pSS cohort studies performed over the past several and recent years, have clearly shown that cryoglobulinaemic vasculitis is the most frequent type of systemic vasculitis accompanying pSS. Antineutrophil cytoplasmic antibody-associated, large and medium vessel vasculitis are described only in sporadic cases. In addition to the overt clinical manifestations of cryoglobulinaemic vasculitis, type II cryoglobulinaemia, glomerulonephritis and purpura have been correlated with increased risk for B-cell non-Hodgkin lymphoma (NHL) in pSS.

Summary
pSS is characterized by autoreactive B and T-cell infiltrates around the epithelial structures of the affected organs, as well as, B-cell hyperreactivity. The latter, is attested by the increased production of autoantibodies, directed against many different organ and nonorgan self-antigens. Vasculitis is a significant and potentially life-threatening complication of the disease depending on the size, localization, histologic type and the pathogenetic mechanisms of the inflammatory process. The potentially irreversible tissue damage, as well as the increased risk for NHL development, prompts the need for early diagnosis and treatment of cryoglobulinaemic vasculitis in pSS.

Keywords
cryoglobulinaemia, lymphomagenesis, Sjögren's syndrome, systemic vasculitis

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a rather common, slowly progressive, systemic autoimmune disease, affecting mainly middle-aged women. Although the salivary and lacrimal glands are the primary targets of the disease, virtually any organ system that has epithelial structures can be affected. The precise pathogenetic mechanisms of the disease are still unknown, but it appears that both arms of the immune response, the innate and adaptive, are aberrantly activated. For the initiation of the disease, the central player appears to be the affected epithelial cell. Indeed, these cells express inappropriately molecules that can break the immune tolerance and initiate adaptive immune responses, such as human leukocyte antigen class II, intracellular autoantigens and costimulatory molecules; they are intrinsically activated, expressing functional Toll-like receptors and producing several cytokines and chemokines, able to create and restore a local inflammatory response (autoimmune epithelitis). As the autoimmune response progresses, effector mechanisms are developed. These mechanisms, that are better understood today, are the major mediators for the tissue injury and, eventually, the clinical manifestations of the disease. Indeed, the two main autoimmune phenomena, observed, when the patient is for the first time seen by a physician are two: First, the lymphocytic infiltration of the exocrine glands, consisting predominantly of autoreactive CD4+ T lymphocytes and B lymphocytes. Second, the polyclonal autoreactive B-cell hyperactivity, leading to germinal center formation in 20–25% of patients and increased

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KEY POINTS

- B-cell lymphoproliferation and immune-complex formation are playing a crucial role in vasculitic development in pSS patients.
- Small vessel vasculitis is the most severe extraglandular manifestation of pSS manifesting as a rash, peripheral neuropathy or glomerulonephritis.
- Cryoglobulinaemic vasculitis is the most frequent systemic vasculitis accompanying pSS, but ANCA-associated, large and medium vessel vasculitis have been also described in sporadic cases.
- Mixed cryoglobulinaemia, glomerulonephritis and purpura in seropositive patients (antinuclear antibodies, rheumatoid factor, anti-Ro/La) are associated with a four-fold increased risk of developing B-cell NHL and are considered as predictive factors of NHL development.

production of autoantibodies against immunoglobulins, cellular, nuclear and other antigens, as well as the activation of type-I interferon pathways [1▪,2]. These autoimmune aberrations generate the extraglandular manifestations that are present in approximately 75% of pSS patients and are currently classified as nonspecific (arthralgias, arthritis, Raynaud's phenomenon and fatigue); periepithelial characterized by lymphocytic invasion of the epithelial tissues in parenchymal organs such as kidneys (interstitial nephritis), lungs (small airways disease) and liver (primary biliary cirrhosis like picture); and mediated by the deposition of immune complexes due to overt B-cell hyperactivity [3]. The latter include purpura, glomerulonephritis and peripheral nerve involvement. Around 5% of pSS patients may develop non-Hodgkin's lymphoma (NHL) [4].

In pSS patients, vasculitis is one of the most clinically important extraglandular manifestations taking many clinical forms. The size and localization of the involved vessels in association with the inflammatory process, account for the wide clinical spectrum, that extends from limited cutaneous lesions to life threatening systemic disease [5,7] (Fig. 1). By far, the most common form of systemic vasculitis in pSS is cryoglobulinaemic vasculitis. Sporadic cases of other forms of vasculitis have also been described [6–8]. The present review discusses the clinical presentation of vasculitis in pSS.

SMALL VESSEL VASCULITIS

Small vessels are the main venue of vasculitic involvement in pSS. The clinical spectrum ranges from local cutaneous disease to a multisystemic disorder with increased morbidity and mortality, especially if remains untreated. Cutaneous vasculitis generally presents as palpable, as it is the case of cryoglobulinaemic vasculitis, or nonpalpable purpura, as it is observed in urticarial vasculitis. Nonpalpable hypergammaglobulinaemic purpura can also be seen frequently, particularly in patients with high levels of serum immunoglobulins, located predominantly in the lower limbs. Skin vasculitis is associated with a higher prevalence of articular and renal disease as well as immunologic features [antinuclear antibodies (ANA), rheumatoid factor, cryoglobulins and anti-Ro/SSA (Sjögren's-syndrome related antigen type A) positivity] [9]. The histopathological examination of skin biopsies from pSS patients with skin vasculitis reveals two different small vessel vasculiti (SVV) patterns. The leukocytoclastic pattern is the most common and it is characterized by polymorphonuclear infiltration, neutrophil fragmentation, extravasation and fibrinoid necrosis of the vessel wall. Unlike leukocytoclastic vasculitis, the lymphocytic pattern, is characterized by a different inflammatory infiltrate containing mainly lymphocytes, plasma cells and

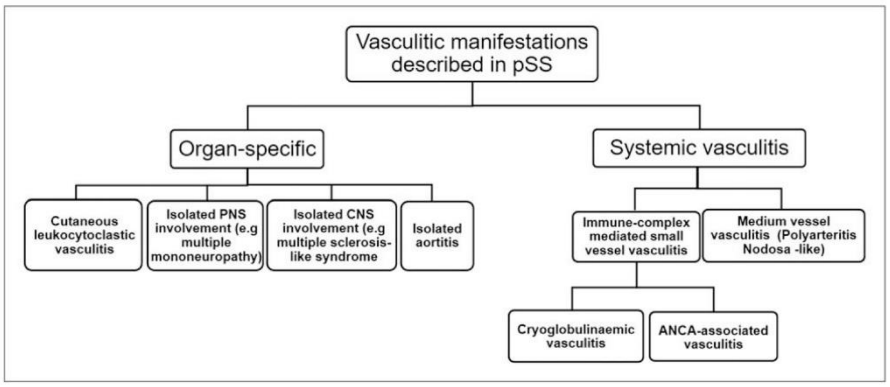

FIGURE 1. Extraglandular manifestations of primary Sjögren's syndrome related to vasculitis.

histiocytes. The vascular wall is intact and the picture is reminiscent of a chronic autoimmune response [10,11▪▪].

Despite the fact that central nervous system (CNS) involvement is extremely rare in pSS, inflammation of small blood vessels has been hypothesized as a potential mechanism that underlies CNS disease in some cases, in the past. These patients usually develop a multiple sclerosis-like syndrome with compatible brain lesions, cranial nerve palsies and myelitis due to inflammation of the spinal cord. It should be noted, however, that cases like these have not been described in recent cohort studies of pSS patients.

Peripheral nervous system (PNS) can also be affected. Distal sensorimotor, small or large fiber sensory and autonomic neuropathy have been described in patients with pSS. Vasculitic neuropathy presents as multiple mononeuropathy and accounts for about 1% of patients. Nerve biopsies commonly reveal epineural vascular inflammation with or without necrosis [12▪,13▪▪].

Anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) has been described in sporadic cases of pSS. Guellec *et al.* in a recent overview of the literature described seven new and 15 previously published cases of coexisting pSS and AAV. These are most commonly p-ANCA associated vasculitis with anti-myeloperoxidase specificity, but isolated c-ANCA, anti-proteinase 3 positive vasculitis (Granulomatosis with Polyangiitis and Eosinophilic Granulomatosis with Polyangiitis) were also described. Glomerulonephritis, lung, skin and PNS involvement were the most prominent, but CNS, small bowel, muscle involvement, ear chondritis and sinuses were also observed. In all cases, pSS diagnosis preceded the clinical onset of AVV [14▪▪].

CRYOGLOBULINAEMIC VASCULITIS

Case report

A 56-year-old female with sicca manifestations and recurrent episodes of parotid gland enlargement the last 5 years, was admitted in the clinic for palpable purpura of the lower limbs, myalgias and painful numbness of the toes. Schirmer's test and Rose Bengal staining of the cornea were positive. Minor salivary gland biopsy disclosed round cell infiltrates with focus score 4. Immunologic and laboratory tests revealed high titer of rheumatoid factor, anti-Ro/SSA and anti-La/SSB (Sjögren's syndrome related antigen type B) autoantibodies, low C4 levels, hypergammaglobulinemia and leukopenia. The diagnosis of pSS was set. Subsequent laboratory evaluation revealed high titer of cryoglobulins (349.9 mg/dl) and the immunofixation demonstrated IgMκ–IgG, type-II cryoglobulinemia. Nerve conduction tests were performed and disclosed sensory motor polyneuropathy. The patient was considered as having cryoglobulinaemic vasculitis in the setting of pSS and was treated with steroids and anti-CD20 B-cell depletion therapy with complete resolution of her symptoms.

Among systemic autoimmune diseases, pSS is ranking first in the prevalence of cryoglobulinaemic vasculitis, that is a rare systemic vasculitis, associated with the presence of serum cryoglobulins (cryoglobulinemia), affecting around 1/100 000 of the general population. It has increased morbidity and is more prevalent in the Mediterranean area. Cryoglobulins, are monoclonal immunoglobulins (type-I) or immune complexes composed mainly by either monoclonal or polyclonal IgM or IgA immunoglobulins with rheumatoid factor activity against polyclonal IgG (mixed type-II and III cryoglobulins respectively). They display the characteristic property to precipitate reversibly at temperatures less than 37 °C (Fig. 2a). Although cryoglobulinaemia is considered as a prerequisite of the disease, not all patients with cryoglobulinemia develop clinical manifestations, suggesting that other factors are also involved in the precipitation of cryoglobulins into the affected tissues. Several causative factors have been associated with cryoglobulinemia and cryoglobulinaemic vasculitis, including infections [hepatitis C virus (HCV), HIV and hepatitis B virus], hematologic malignancies and autoimmune diseases. The most common causes of cryoglobulinaemic vasculitis are HCV infection and pSS. A systematic study to address the prevalence of cryoglobulinaemic vasculitis in consecutive patients with pSS does not exist, but cohort studies suggest that it may occur in 5–10% of pSS patients. Patients with cryoglobulinaemic vasculitis or cryoglobulinemia are at high risk for B-cell origin lymphoproliferative disorders, implying that may serve as predictors of lymphoma development and therefore these patients should be closely followed-up. The pathogenetic mechanisms, mediating cryoglobulinaemic vasculitis development are partially understood. However, B cells appear to be a central player, since they have been shown to clonally expand and produce monoclonal IgM rheumatoid factor that forms cold-precipitable immune complexes responsible for vasculitis. These clonal B cells display specific characteristics such as low expression of CD21 and features of functional exhaustion (CD19highCD11c+CD95+CD62Llow/neg) [15,16▪▪,17]. For pSS, cryoglobulins are considered to rise following a chronic antigenic stimulation due to either a yet unknown viral antigen or

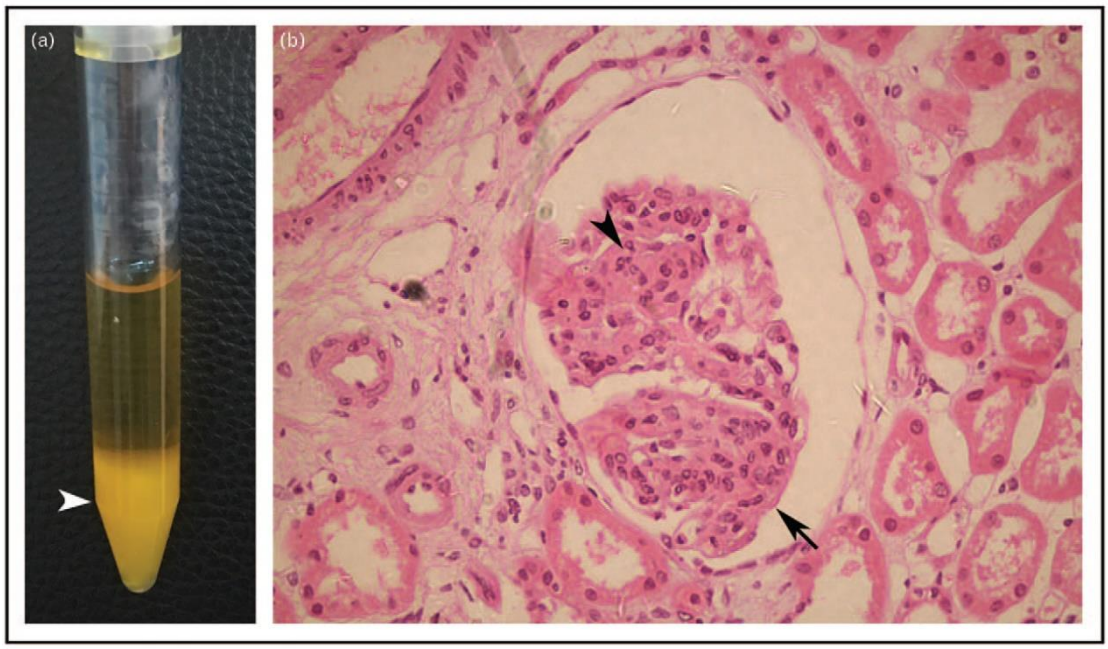

FIGURE 2. Cryoglobulins and cryoglobulinaemic membranoproliferative glomerulonephritis in primary Sjögren's syndrome. (a) Serum cryoglobulins precipitated (arrowhead) after incubation at 4 °C for 7 days from a primary Sjögren's syndrome patient with cryoglobulinaemic vasculitis. (b) Cryoglobulinemic membranoproliferative glomerulonephritis in primary Sjögren's syndrome. Expansion of the mesangial matrix and increased cellularity of the mesangium (arrowhead) in combination with thickening and reduplication of the glomerular basement membrane producing the typical histologic picture of 'double contour' or 'tram tracking' (arrow). (Hematoxylin and eosin ×400) (Image kindly provided by Professor Lydia Nakopoulou, First Department of Pathology, Medical School of Athens).

an autoantigen, whereas the production site of cryoglobulins appears to be in the inflamed salivary epithelium [18]. Significantly, the association of both HCV hepatitis and pSS with the development of B-cell lymphomas, using similar immunoglobulin VDJ (variable, diversity and joining) sequences [19] further signifies that cryoglobulinaemic vasculitis and cryoglobulins are probably a common mechanism, preceding lymphoma.

Cryoglobulinaemic vasculitis has a wide spectrum of clinical and laboratory features (Table 1). The majority of cryoglobulinaemic vasculitis patients in the setting of pSS complain of fatigue (80–90%) but the presenting feature of this type of vasculitis is palpable purpura (70–90%) usually at the lower limbs, leaving a brownish pigmentation as it resides. Large skin ulcers typically above the malleoli, digital necrosis, lullae and livedo racemosa may also occur. A distal painful sensory or sensorimotor polyneuropathy due to vasculitis of the vasa nervosum is the most frequent neurologic manifestation of the disease (60–70%). Axonopathy and mononeuritis multiplex have been also described [12▪,20]. Renal disease occurs in 20–35% of cryoglobulinaemic vasculitis patients and ranges from asymptomatic to acute renal failure. Proteinuria is the most common clinical expression with membranoproliferative glomerulonephritis (Fig. 2b) presenting subendothelial deposits of cryoglobulins being the most common histological finding (80%) [21]. Type-II cryoglobulinaemia as well as high serum cryoglobulin concentration have been proposed as risk factors for renal disease independently to vasculitic involvement [22▪].

MEDIUM VESSEL VASCULITIS

Unlike SVV, inflammation of medium vessels is very infrequent in pSS occurring in less than 5% of Sjögren's syndrome patients with vasculitic involvement and, thus, only a few case reports were found in the literature. Acute necrotizing vasculitis resembling polyarteritis nodosa (PAN) in pSS patients was first described by Tsokos *et al.* in 1987. The disease was predominantly localized in internal organs and associated with life-threatening symptoms. Histologically, the vascular wall exhibited heavy infiltration with acute and to a lesser degree, with chronic inflammatory cells. Fibrinoid necrosis was present, but the patients lacked the characteristic

Table 1. Organ involvement and laboratory findings related to cryoglobulinaemic vasculitis in primary Sjögren's syndrome patients

Clinical manifestations
Constitutional-Glandular
Fatigue
Fever
Dry eyes
Dry mouth
Dyspareunia
Lymphadenopathy
Parotid gland enlargement
Cutaneous
Purpura
Necrosis
Ulcers
Livedo reticularis
Raynaud's phenomenon
Musculoskeletal
Arthralgias/myalgias
Arthritis (symmetric nondeforming, knees and hands >elbows, ankles)
Peripheral neuropathy
Distal sensory or sensorimotor polyneuropathy
Axonopathy
Mononeuritis multiplex
CNS involvement
Multiple sclerosis-like syndrome
Cranial nerve involvement
Myelitis
Renal involvement
Glomerulonephritis (mesangial, membranoproliferative or membranous)
Proteinuria
Microscopic hematuria
Gastrointestinal
Lymphoma
Laboratory findings
ANA
Autoantibodies to Ro/La ribonucleoproteins
RF
Cryoglobulins (mixed IgMk)
Low C4
Hypergammaglobulinaemia
Leukopenia

ANA, antinuclear antibodies; CNS, central nervous system; RF, rheumatoid factor.

aneurysmal formation [11▪▪]. Similarly, another group found that two of 52 pSS patients had vasculitis of medium sized arteries. One had coexistent PAN and one necrotizing vasculitis of the pancreatic and mesenteric arteries [9]. Another form of vasculitic involvement seen in Sjögren's syndrome is endarteritis obliterans, a noninflammatory obstructive vasculitis affecting medium-sized vessels. This condition presents with fibrous thickening of the intima, leading to variable lumen stenosis with recanalization and thus, impairment of blood flow, causing ischemia/infarction of the dependent tissues. Residual mononuclear cells may be seen in the adventitia suggesting that this type of vasculitis may in fact represent the healing stage of preexisting acute vasculitis [11▪▪].

LARGE VESSEL VASCULITIS

Large vessel vasculitis (LVV) represents a heterogeneous group of rare diseases characterized by intramural inflammation of the aorta, its major branches and extremity arteries. So far, data suggest that the inflammatory invasion, stimulated by an unknown trigger, commences from the adventitia at the level of vasa vasorum and progresses to the intima leading to segmental stenosis, occlusion and aneurysm formation [23]. Several molecular mechanisms, also involved in the pathogenesis of Sjögren's syndrome, including matrix metalloproteinase activation, c-Jun N-terminal kinase, nuclear factor kappa-light-chain-enhancer of activated B cells and transforming growth factor beta signaling pathways are implicated in the initiation and progression of aortic aneurysms and aortic dissection [24]. Significantly, while LVVs are mainly regarded as T-cell mediated diseases, the immunohistochemical analysis of aortic tissue samples of LVV patients, who underwent aortic aneurysm surgery, revealed massive B-cell infiltrates organized into ectopic germinal centers, within the affected arterial wall [25▪▪]. In pSS patients, LVV is rare and only a few cases have been reported in the literature. The first case of inflammatory abdominal aortic aneurysm in pSS patient with positive anti-Ro and anti-La autoantibodies was reported by Ghinoi *et al.* [26], whereas more recently Heper *et al.* [27] described a case of seronegative pSS patient with pleural and pericardial effusions, ascites and ascending aortic aneurysm with increased uptake of 18-Fluorodeoxyglucose (FDG) in FDG–PET. A recent retrospective cohort study of 10941 Chinese Sjögren's syndrome patients showed that Sjögren's syndrome patients exhibit not only increased prevalence of cardiovascular risk factors, including hypertension, hypertriglyceridemia and early atheromatosis as previously shown [28], but also increased risk of aortic aneurysm and aortic dissection [29▪▪]. This clinical observation has not been described in non-Asian patients with pSS, and remains an open question, that can

be addressed using noninvasive methods of vascular inflammation, including vascular ultrasound or PET–FDG [23]. The common signaling pathways and the emerging role of B cell hyperreactivity in aortic aneurysms, could suggest that chronic aortitis might be a manifestation of a systemic autoimmune process rather than an aberrant local reaction to atherosclerosis. Cerebral LVV has also been described in strongly anti-Ro and anti-La positive pSS patients, but an etiologic linkage between the two diseases cannot be supported yet [30].

PROGNOSIS OF VASCULITIS IN SJÖGREN'S SYNDROME

GEMESS Study Group examined the clinical features in 1010 Spanish pSS patients and found that increased frequency of vasculitis was associated with younger age (disease onset <35 years), long-term (>10 years) duration, as well as positive ANA, rheumatoid factor and anti-Ro/La [31]. Type-II cryoglobulinaemia is present in approximately 5–20% of pSS patients and has been associated with high prevalence of extraglandular disease, increased levels of rheumatoid factor, anti-Ro/SSA and low serum C4 [32▪]. Tzioufas *et al.* [33▪▪] were the first to demonstrate that mixed monoclonal cryoglobulinaemia is a predictive factor for lymphoma development. Three years later Voulgarelis *et al.* found that NHL patients exhibit increased prevalence of skin vasculitis (11%). Most of these patients had increased levels of rheumatoid factor and cryoglobulins and low C4 serum levels [34]. Cryoglobulinaemic vasculitis is a major factor of increased morbidity in pSS. Despite the fact that most patients experience a slowly progressive and benign course, 35% present with moderate to severe disease course involving internal organs. The 10-year survival rate in mixed cryoglobulinaemic vasculitis is 56%. The worse prognosis is conferred by glomerulonephritis especially in older aged (≥60 years), male patients. Higher serum creatinine levels and increased proteinuria at cryoglobulinaemic vasculitis diagnosis predispose to kidney failure and death [16▪▪,35▪,36]. The correlation between cryoglobulinaemic vasculitis and lymphoma development that has been discussed above, has an additive effect in the mortality of pSS and cryoglobulinaemic vasculitis patients. Symptomatic lymphomas are reported in 5–20% of cryoglobulinaemic vasculitis patients within 10 years of diagnosis, with B-cell lymphomas of the mucosa associated lymphoid tissue type being the most common. Immunosuppression due to chronic immunosuppressive therapy accounts for serious infections, but this constitutes a less frequent cause of death in these patients [37,38▪▪,39].

TREATMENT OF PRIMARY SJÖGREN'S SYNDROME-RELATED VASCULITIS

As previously reported systemic vasculitis is a potentially life threatening disease especially, if remains untreated. For immune-complex mediated systemic vasculitis (AAV and cryoglobulinaemic vasculitis) corticosteroids are the gold-standard of initial management especially in patients with severe multiorgan involvement. Steroids should be tapered quickly and there is no rule for chronic therapy. Other immunosuppressive agents including cyclophosphamide, azathioprine and methotrexate are also used as induction or maintenance therapy especially in patients with renal or PNS involvement with variable response. It should be noted, however, that chronic exposure of patients with pSS to alkylating agents may increase the potency of lymphomagenesis.

Anti-CD20 treatment (Rituximab), targeting B cells, has shown a very good efficacy in treating mixed cryoglobulinaemic vasculitis (aiming to limit the production of cryoglobulins) and AAV and should be considered in all patients with severe to moderate disease [16▪▪,20,40,41]. In patients with hyperviscosity syndrome due to increased concentration of cryoglobulins, as well as rapidly glomerulonephritis (mainly seen in AAV) plasma exchange can serve as an additional therapeutic option [42,43]. Potential therapies for refractory cryoglobulinaemic vasculitis cases are mycophenolate mofetil and belimumab while tumor necrosis factor (TNF) inhibitors have been proved ineffective [44]. Colchicine has been reported to have favorable effect on purpura and leg ulcers [45]. High dose of steroids are the cornerstone of LVV therapy. Methotrexate, mycophenolate mofetil, azathioprine and hydroxychloroquine have been used as steroid sparing agents. For refractory disease, steroid dependent or resistant cases the anti-IL6R biologic agent (Tocilizumab) is recommended as an effective alternative. Anti-TNF agents can be also considered in recalcitrant disease despite the conventional therapy [46▪▪].

CONCLUSION AND FUTURE PERSPECTIVES

Vasculitis is a severe extraglandular manifestation of pSS contributing to the increased morbidity and mortality of the disease. Mixed cryoglobulinaemia, glomerulonephritis and purpura are associated with a four-fold increased risk of NHL development. The properties of the disease can serve as an excellent model to get further insights into the pathogenetic mechanisms underlying, autoreactive B cells,

autoantibody production and the transition from benign B-cell polyclonality to malignant lymphoma. Further studies aiming to characterize B cells and mechanisms mediating their activation/differentiation/transition; discover and validate biomarkers predicting the development of cryoglobulinaemic vasculitis; and the harmonization and stratification of cryoglobulinaemic vasculitis patients, are anticipated to provide relevant insights into autoimmunity and hematologic malignancies and reveal novel therapeutic targets.

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Conflicts of interest

There are no conflicts of interest.

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- ■ of special interest
- ■■ of outstanding interest

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Cryoglobulinemic vasculitis in primary Sjögren's Syndrome: Clinical presentation, association with lymphoma and comparison with Hepatitis C-related disease

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ABSTRACT

Objective: To describe the clinical spectrum of cryoglobulinemic vasculitis (CV) in primary Sjögren's syndrome (pSS), investigate its relation to lymphoma and identify the differences with hepatitis C virus (HCV) related CV.
Methods: From a multicentre study population of consecutive pSS patients, those who had been evaluated for cryoglobulins and fulfilled the 2011 classification criteria for CV were identified retrospectively. pSS-CV patients were matched with pSS patients without cryoglobulins (1:2) and HCV-CV patients (1:1). Clinical, laboratory and outcome features were analyzed. A data driven logistic regression model was applied for pSS-CV patients and their pSS cryoglobulin negative controls to identify independent features associated with lymphoma.
Results: 1083 pSS patients were tested for cryoglobulins. 115 (10.6%) had cryoglobulinemia and 71 (6.5%) fulfilled the classification criteria for CV. pSS-CV patients had higher frequency of extraglandular manifestations and lymphoma (OR=9.87, 95% CI: 4.7–20.9) compared to pSS patients without cryoglobulins. Purpura was the commonest vasculitic manifestation (90%), presenting at disease onset in 39% of patients. One third of pSS-CV patients developed B-cell lymphoma within the first 5 years of CV course, with cryoglobulinemia being the strongest independent lymphoma associated feature. Compared to HCV-CV patients, pSS-CV individuals displayed more frequently lymphadenopathy, type II IgMk cryoglobulins and lymphoma (OR = 6.12, 95% CI: 2.7–14.4) and less frequently C4 hypocomplementemia and peripheral neuropathy.
Conclusion: pSS-CV has a severe clinical course, overshadowing the typical clinical manifestations of pSS and higher risk for early lymphoma development compared to HCV related CV. Though infrequent, pSS-CV constitutes a distinct severe clinical phenotype of pSS.

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Introduction

Cryoglobulinemic vasculitis (CV) is a life threatening immune complex mediated small vessel vasculitis, involving primarily the skin, kidneys, and peripheral nerves, leading to end stage organ/tissue damage, if untreated [1,2]. It can be of infectious origin, with hepatitis C virus (HCV) infection being the most common cause, or associated with autoimmune diseases where the most frequent underlying condition is primary Sjögren's syndrome (pSS). As new, effective therapeutic modalities for the management of HCV infection keep emerging, pSS will soon become the leading cause of CV [2,3]. CV is associated with the presence of serum cryoglobulins of which, type II containing an IgMκ monoclonal rheumatoid factor (mRF) predominate in pSS, while both type II and III are detected in HCV and other autoimmune diseases. Previous studies have clearly shown that type II cryoglobulinemia in pSS is associated with more systemic manifestations, mainly vasculitis [4], and higher risk for future lymphoma development [5]. Thus, cryoglobulinemia is at the crossroad of the two most serious complications of pSS, that is, systemic vasculitis and B-cell non- Hodgkin's lymphoma (NHL). With the advent of new treatments, targeting successfully B-cells and NHL [6], the investigation and mapping of the clinical spectrum of pSS-CV may provide a concise strategic plan for early diagnosis and treatment for this subset of pSS. To this end, older studies have described the clinical picture of pSS patients in association with cryoglobulinemia [7–9], while after the introduction of CV criteria [10,11] some studies have focused on CV [12–16]. However, even after the application of CV classification criteria, the clinical picture of pSS-CV is still obscured due to the fact that many of the previous studies have a small number of pSS patients.

Herein, we present the clinical phenotype of CV in an integrated study population of Greek-Italian pSS patients, investigate the possible role of CV and cryoglobulinemia in NHL development in pSS and compare the clinical manifestations of pSS-CV with HCV-CV.

Patients and methods

Study design

This is a retrospective, matched case-control study in a multicenter population of consecutive pSS patients who fulfilled the 2016 ACR/EULAR classification criteria [17] and were followed up from May 1984 until March 2019, in 5 centers from Greece and Italy (University of **U**dine, Pisa, **A**thens, **H**arokopio, **I**oannina) (**UPAHI** group). The study was approved by the local ethical committees of all the Institutions involved, after obtaining patients' informed consent and in compliance to general data protection regulations (GDPR). One thousand eighty-three patients had been evaluated for serum cryoglobulins and 71 of them fulfilled the 2011 classification criteria for CV [10]. Cryoglobulins were evaluated after blood collection, quantitation, immunodiffusion and immunofixation, as described previously [4]. All pSS patients were HCV-RNA negative. The cumulative clinical, laboratory and histologic data of pSS-CV patients were compared with two control groups: a) pSS patients, repeatedly negative for serum cryoglobulins, matched (1:2 ratio) according to gender, age at pSS onset and disease duration from pSS onset and b) patients with HCV related CV, being RNA positive at CV diagnosis and without any associated autoimmune rheumatic disease, matched (1:1) according to age and gender. All patients with HCV related CV were diagnosed, treated and followed-up at the Infectious Disease Unit, Department of Clinical and Biomedical Sciences Hospita L. Sacco, Milan, Italy. In addition, pSS cryoglobulin-positive patients who did not fulfill the 2011 CV classification criteria, were compared with a pSS cryoglobulin negative control group, matched (1:2 ratio) according to gender, age and disease duration from pSS onset. All the laboratory, objective tests or minor salivary gland biopsy of pSS patients, were performed in the context of standard of care, according to physicians' judgment. pSS onset was defined as the year when the patient recalled the first disease related manifestation, such as Raynaud's phenomenon, arthritis, sicca symptoms, salivary gland enlargement or purpura. CV onset was defined as the time point of the appearance of the first CV related manifestation, according to the 2011 classification criteria. Groups were compared on the basis of cumulative clinical (dry mouth, dry eyes, salivary gland enlargement, Raynaud's phenomenon, lymphadenopathy, arthralgia/myalgia, arthritis, palpable purpura, liver involvement, kidney involvement, central and peripheral nervous system involvement, lymphoma), laboratory (anti Ro/SSA antibodies, anti La/SSB antibodies, rheumatoid factor, cryoglobulinemia, low serum C4 complement levels, monoclonal gammopathy) and histologic (focus score, germinal centers) features. Systemic organ involvement was based on the ESSDAI definitions and/or biopsy specimens [18]. Fatigue, dryness and pain were assessed as defined by the ESSPRI [19]. Since different pathogenetic mechanisms are operating in glandular and extraglandular manifestations of pSS, they have been classified as glandular (dry mouth, dry eyes, salivary gland swelling), non-specific manifestations (fatigue, arthralgia/myalgia, arthritis, Raynaud's phenomenon), peri-epithelial (interstitial nephritis, primary biliary cholangitis, small airways disease), immune complex mediated (extra-epithelial) (purpura, skin ulcers, glomerulonephritis, vasculitic involvement of peripheral and/or central nervous system) and NHL [20].

Statistical and data driven analysis

Statistical analysis for categorical data was performed by $\chi 2$ test with Yates correction or Fisher exact when cell counts <5 patients and for numerical data *t*-test or Mann-Whitney, after Shapiro-Wilk normality test. In order to handle the multiple comparison testing, p-values have been also adjusted with Bonferroni correction. The Fast-Correlation based feature selection (FCBF) algorithm was applied on the dataset of pSS-CV patients and their pSS cryoglobulin negative controls, to identify potentially independent variables associated with lymphoma [21]. The FCBF preselection algorithm is a correlation based tool identifying, among several potentially independent variables, those with the weakest association amongst them and the strongest correlation with the outcome of interest that is NHL. Subsequently, the strongest preselected group of the FCBC derived potentially independent variables, has been used for constructing a binary multivariable logistic regression model to identify independent variables/features associated with lymphoma. The implementation of the FCBF-based multivariable logistic regression approach along with the statistical analysis was performed using Python 3.6 and GraphPad 7.0a.

Based on the post hoc sample size and study power calculation conducted according to the Fleiss method, assuming 90% study power and 95% two-sided levels of confidence, the present study sample size could detect an effect size (Odds Ratio) of 5.00 between patient groups (EpiInfo, CDC, Atlanta, Georgia, USA).

Results

Patient characteristics

Serum cryoglobulins were detected in 115/1083 patients (10.6%) of whom 71 (61.7%) fulfilled the 2011 CV classification criteria, while 44 (38.3%) had pSS with cryoglobulinemia but did not meet the CV criteria. Early ($\leq$ 35 years) pSS onset had 19.7% (n = 14/71) of pSS-CV patients, while 12.7% (n = 9/71) had late ($\geq$ 65 years) pSS onset. Among pSS-CV patients, 97% were females (n = 69/71) and 3% males (n = 2/71). The median age of pSS-CV patients, calculated at pSS onset, was 50 years (range: 21–75). The median duration from pSS onset in pSS-CV patients was 16 years (range: 0–37). None of the pSS or RNA-HCV positive control patients fulfilled criteria for another systemic autoimmune disease. pSS cryoglobulin positive patients without

vasculitis were also predominantly females [95.5%, (n = 42/44) vs 4.5%, (n = 2/44)] with a median age at pSS onset of 50 years (range: 11–79) and median disease duration from pSS onset of 13 years (range: 0–42).

The clinical phenotype of pSS-cryoglobulin positive patients with and without CV

The clinical picture of 71 pSS-CV patients was compared with that of 141 pSS cryoglobulin negative matched control patients. pSS-CV patients exhibited higher frequency of fatigue (59.2% vs 43%, p = 0.041, OR = 1.92, 95% CI: 1.08–3.52), Raynaud's phenomenon (47.9% vs 32.6%, p = 0.044, OR = 1.89, 95% CI: 1.06–3.39), salivary gland enlargement (53.6% vs 33.3%, p = 0.007, OR = 2.31, 95% CI: 1.29–4.21) and interstitial renal disease (10% vs 1.5%, p = 0.007, OR = 7.55, 95% CI: 1.59–36.4), compared to pSS-cryoglobulin negative patients respectively. No difference was found in sicca manifestations between the 2 groups. As anticipated, pSS-CV patients had increased prevalence of extra-epithelial manifestations of a vasculitic origin, including purpura (90.1% vs 14.9%, $p < 0.0001$, OR = 52.24, 95% CI: 21.43–125.4), vasculitic ulcers (12.7% vs 0.71%, $p < 0.001$, OR = 20.32, 95% CI: 3.17–224.4), peripheral nervous system vasculitic involvement (25.4% vs 1.5%, $p < 0.0001$, OR = 21.74, 95% CI: 5.12–96.01), glomerulonephritis, mainly of membranoproliferative type, (11.4% vs 0.71%, $p < 0.001$, OR = 18.06, 95% CI: 2.64–201.8) and lymphadenopathy (31% vs 7.1%, $p < 0.001$, OR = 5.88, 95% CI: 2.63–12.99). NHL, mainly of MALT type, was more frequent in the pSS-CV group (47.9% vs 8.5%, $p < 0.0001$, OR = 9.87, 95% CI: 4.7–20.9). Minor salivary gland biopsies of pSS-CV patients displayed a higher proportion of germinal centers (35% vs 11.3%, p = 0.043, OR = 4.21, 95% CI: 1.20–13.45) and a higher focus score [median: 2.05 (range: 0–9) vs 1.45 (range: 0–7)] compared to pSS cryoglobulin negative controls. The laboratory analysis disclosed that almost all pSS-CV patients had positive rheumatoid factor (95.7% vs 61.3, $p < 0.0001$, OR = 14.09, 95% CI: 4.58–44.33) as well as low C4 complement levels (88.6% vs 31.5, $p < 0.0001$, OR = 16.82, 95% CI: 7.47–35.57). Monoclonal gammopathy was also more prevalent in the pSS-CV group (45.5% vs 7.6%, $p < 0.0001$, OR = 10.17, 95% CI: 4.41–22.17). The presence of anti-Ro/SSA and anti-La/SSB autoantibodies was comparable between the two groups. A more detailed comparison of the clinical, laboratory, serological and histologic features between the 2 groups are presented in Table 1. After applying Bonferroni correction, fatigue,

Table 1
Comparison of clinical, laboratory and histologic features between pSS-CV and pSS patients with negative serum cryoglobulins.

	pSS-CV	pSS-Cryo negative	*P*-value	*P*-value*
Number of patients	71	141		
Gender (female%)(n)	97.2 (69/71)	96.5 (136/141)	1	1
Disease duration (from SS onset _years)	16 (range: 0–36)	15 (range: 0–34)	0.720	1
Media age (years)	50 (range: 21–75)	50 (range: 20–77)	0.887	1
Clinical Features% (n)				
Dry mouth	97.2 (69/71)	93.6 (132/141)	0.342	1
Dry eyes	98.6 (70/71)	92.9 (131/141)	0.104	1
SGE	53.6 (37/69)	33.3 (47/141)	0.007	0.252
Lymphadenopathy	31 (22/71)	7.1 (10/141)	<0.0001	<0.001
Fatigue	59.2 (42/71)	43(55/128)	0.041	1
Arthralgia/myalgia	71.8 (51/71)	63.8 (90/141)	0.312	1
Arthritis	25.7 (18/70)	18.3 (23/126)	0.295	1
Myositis	0(0/71)	1.4 (2/141)	0.552	1
Raynaud's phenomenon	47.9 (34/71)	32.6 (46/141)	0.044	1
Purpura	90.1 (64/71)	14.9 (21/141)	<0.0001	<0.0001
Vasculitic ulcer	12.7 (9/71)	0.71 (1/141)	<0.001	0.009
PNS	25.4 (18/71)	1.5 (2/130)	<0.0001	<0.0001
CNS	2.8 (2/71)	2.3 (3/129)	1	1
Liver				
Sclerosing Cholangitis	0(0/71)	0(0/141)	1	1
AIH	1.4 (1/71)	1.4 (2/141)	1	1
PBC	1.4 (1/71)	2.8 (4/141)	0.666	1
Lung				
ILD	8.6 (6/70)	6.8 (9/133)	0.853	1
Small airways disease	13(7/54)	6.6 (8/121)	0.273	1
Kidney				
Interstitial renal disease	10 (7/70)	1.5 (2/138)	0.007	1
Glomerulonephritis	11.4 (8/70)	0.71 (1/141)	<0.001	0.026
Splenomegaly	2.8 (2/71)	10.8 (8/74)	0.260	1
Lymphoma	47.9 (34/71)	8.5 (12/141)	<0.0001	<0.0001
Biological features% (n)				
Focus score (median)	2.05 (34/71)	1.42 (78/141)	0.025	0.9
Germinal centers	35 (7/20)	11.3 (6/53)	0.043	1
Anti-Ro	90 (63/70)	85(119/140)	0.429	1
Anti-La	60 (42/70)	46(64/139)	0.078	1
Anti-Ro/La	88.4 (61/69)	83.6 (117/140)	0.472	1
RF	95.7 (67/70)	61.3 (84/137)	<0.0001	<0.0001
Low C4	88.6 (62/70)	31.5 (41/130)	<0.0001	<0.0001
Hypergammaglobulinemia	69.7 (46/66)	56.9 (74/130)	0.114	1
Monoclonal gammopathy	45.5 (30/66)	7.6 (10/132)	<0.0001	<0.0001
Leukopenia	17.4 (12/69)	10 (14/140)	0.193	1
Thrombocytopenia	3(2/67)	2.2 (3/134)	1	1

Cryo: cryoglobulinemia, SGE: salivary gland enlargement, PNS: peripheral nervous system, CNS: central nervous system, AIH: autoimmune hepatitis, PBC: primary biliary cirrhosis, ILD: interstitial lung disease, RF: rheumatoid factor.
*: Bonferroni adjusted p-value.

Raynaud's phenomenon, salivary gland enlargement, interstitial renal disease, focus score and germinal centers were found with no statistically significant difference between the 2 groups (Table 1).

The FCBF algorithm, that constitutes the data driven analytic approach, performed analysis of 36 features/variables and disclosed 8 strong potentially independent variables [lymphadenopathy, cryoglobulinemia, positive rheumatoid factor (RF), salivary gland enlargement (SGE), dry mouth, arthritis, CNS involvement and lung-bronchocentric involvement accounting for small airways disease]. Narrowing of the analysis, using a combined FCBF/multivariable logistic regression model, showed that lymphadenopathy, cryoglobulinemia and positive RF are independent lymphoma associated features (supplementary Table 1). The performance of the model disclosed 70.6% sensitivity, 94.7% specificity and 84.5% accuracy area under the curve (AUC = 85.5%), after a 10-fold cross validation approach (supplementary Figure 1).

The most common presenting manifestations, according to the 2011 CV classification criteria, of pSS-CV patients were arthralgia/myalgia (49%), followed by arthritis (43.7%) and purpura (39.1%) (Fig. 1A). More than half of the patients (58.2%) with CV developed the first CV specific manifestation within the first year of pSS onset (Fig. 1B). In one third of pSS-CV patients (33.3%), lymphoma was observed within 5 years of CV onset, while in 13.3% of them, the appearance of lymphoma was a late sequel (>15 years of CV duration) (Fig. 1C).

1A) Most common presenting clinical manifestations of CV after applying the 2011 CV classification criteria, 1B) Chronological presentation of first CV related manifestation after pSS onset, 1C) Time distribution of lymphoma development during the course of CV.

The comparison between pSS cryoglobulin positive patients without CV and pSS cryoglobulin negative controls is presented in Table 2. The pSS cryoglobulin positive group without CV, had a higher frequency of monoclonal gammopathy (22% vs 7.3%, p = 0.040, OR = 3.56, 95% CI: 1.16–11.16) and NHL (29.5% VS 5.9%, P<0.001, OR = 6.26, 95% CI: 2.13–17.67). Data regarding lymphoma and type of cryoglobulins were available in 27 of 44 pSS cryoglobulin positive patients without CV. Type II cryoglobulinemia, containing an IgMκ monoclonal RF, was present in 87.5% (n = 7/8) of pSS patients with lymphoma and 47.3% (n = 9/19) in those without, while type III cryoglobulinemia was more prevalent in non-lymphoma (52.7%, n = 10/19) compared to lymphoma (12.5%, n = 1/8) pSS patients. After Bonferroni adjustment, monoclonal gammopathy was found with no statistically significant difference between the 2 groups (Table 2).

Overall, the 71 pSS-CV patients with either mild/non-specific or serious manifestations were treated as follows: corticosteroids (CS) 86%, hydroxychloroquine (HCQ) 80.3%, azathioprine (AZA) 21.1%, methotrexate (MTX) 22.5%, cyclophosphamide (CyC) 8.5%, rituximab (RTX) 18.3% and plasmapheresis 7%. Notably, pSS-CV patients without lymphoma received more frequently AZA, MTX and CyC compared to those with lymphoma (32.4% vs 8.8%, 27% vs 17.6% and

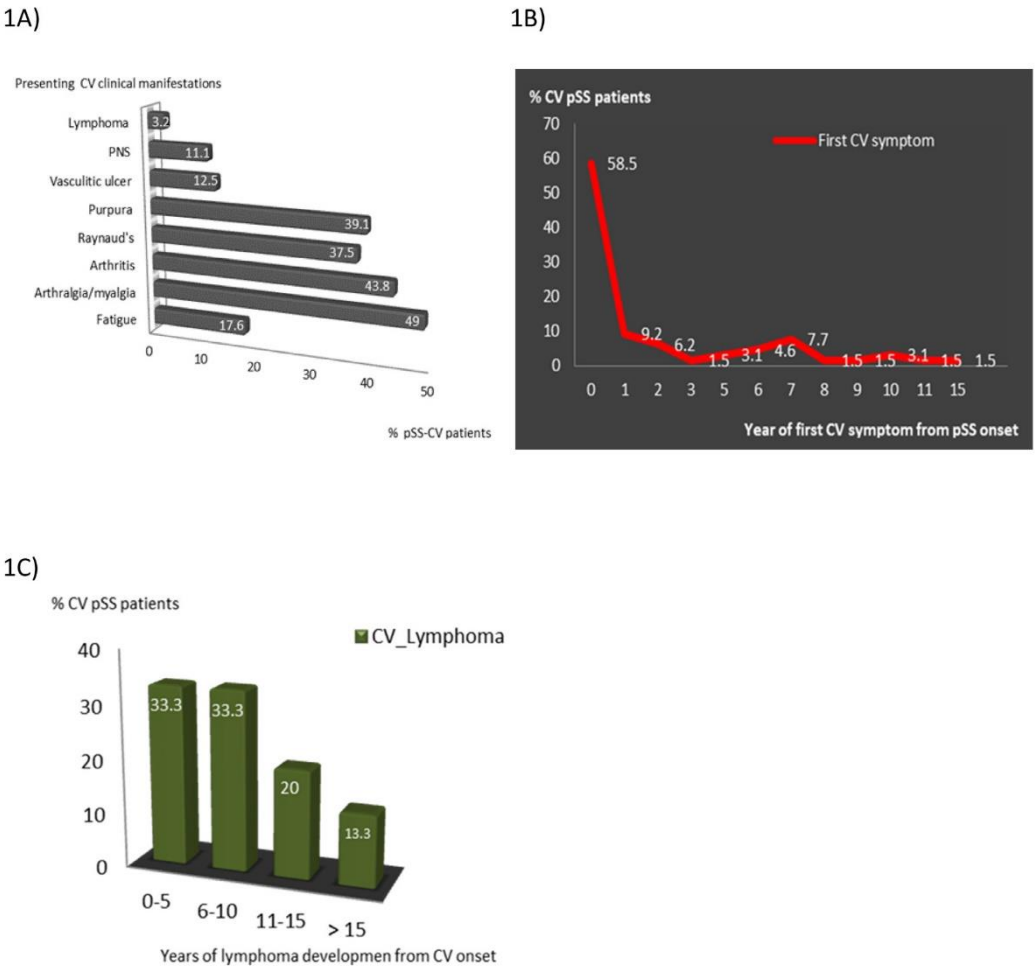

Fig. 1. The clinical course of pSS-CV patients.

Table 2
Clinical, laboratory and histologic features of cryoglobulin positive pSS patients without CV compared to pSS cryoglobulin negative patients.

	pSS-Cryo without CV	pSS-Cryo negative	*P*-value	*P*-value*
Number of patients	44	84		
Gender (female)% (n)	95.5 (42/44)	95.2 (80/84)	0.700	1
Median age (years)	50 (range:11–79)	51	0.695	1
Median disease duration (from SS onset _years)	13 (range:0–42)	11.5	0.628	1
Clinical features% (n)				
Dry mouth	95.5 (42/44)	95.2 (80/84)	1	1
Dry eyes	97.7 (43/44)	95.2 (80/84)	0.659	1
SGE	48.8 (21/43)	29.8 (25/84)	0.054	1
Lymphadenopathy	13.6 (6/44)	6 (5/84)	0.253	1
Fatigue	36.8 (14/38)	43.6 (37/80)	0.444	1
Arthralgia/myalgia	56.8 (25/44)	53.6 (45/84)	0.870	1
Arthritis	10.8 (4/38)	8.9 (7/79)	0.746	1
Myositis	0 (0/44)	0 (0/84)	1	1
Raynaud's phenomenon	38.6 (17/44)	28.6 (24/84)	0.337	1
Purpura	6.8 (3/44)	15.5 (13/84)	0.253	1
Vasculitic ulcer	2.3 (1/44)	1.2 (1/84)	1	1
PNS	4.5 (2/44)	3.6 (3/82)	1	1
CNS	2.3 (1/44)	2.4 (2/82)	1	1
Liver				
Sclerosing Cholangitis	0 (0/44)	2.4 (2/784)	0.545	1
AIH	0 (0/44)	0 (0/84)	1	1
PBC	0(0/42)	3.6 (3/84)	0.550	1
Lung				
ILD	9.1 (4/44)	4.9 (4/81)	0.450	1
Small airways disease	0 (0/40)	6.5 (5/77)	0.163	1
Kidney				
Interstitial renal disease	4.5 (2/44)	1.2 (1/83)	0.275	1
Glomerulonephritis	2.3 (1/44)	1.2 (1/83)	1	1
Splenomegaly	0 (0/44)	0 (0/84)	1	1
Lymphoma	29.5 (13/44)	6(5/84)	<0.001	0.023
Biological features% (n)				
Anti-Ro/SSA	70.5 (31/44)	71.4 (60/84)	0.928	1
Anti-La/SSB	25 (11/44)	41.7 (35/84)	0.094	1
RF	70.7 (29/41)	53(44/83)	0.090	1
Low C4	53.6 (22/41)	36.3 (29/80)	0.100	1
Hypergammaglobulinemia	59(23/39)	55(44/80)	0.831	1
Monoclonal gammopathy	22 (9/41)	7.3 (6/82)	0.040	1
Leukopenia	4.5 (2/44)	8.3 (7/84)	0.717	1
			0.122	1

Cryo: cryoglobulinemia, SGE: salivary gland enlargement, PNS: peripheral nervous system, CNS: central nervous system, AIH: autoimmune hepatitis, PBC: primary biliary cirrhosis, ILD: interstitial lung disease, RF: rheumatoid factor.
*: Bonferroni adjusted p-value.

10.8% vs 5.9 respectively). The 141 pSS cryoglobulin negative controls have received: CS 31.4%, HCQ 46.4%, AZA 4.3%, MTX 7.1%, CyC 0.7% and RTX 4.3%. Interestingly, pSS-CV patients with serious manifestations such as glomerulonephritis, peripheral neuropathy and vasculitic ulcers were treated with steroids, hydroxychloroquine, B cell depletion therapy and very few cases with cyclophosphamide or plasmapheresis. A more detailed description of treatment is presented in supplementary Table 2. The therapeutic regimen of the 44 cryoglobulin positive without CV pSS patients versus (vs) their controls included: CS 38.6% vs 39%, HCQ 45.5% vs 65.9%, AZA 9.1% vs 6.1%, MTX 2.3% vs 7.3%, CyC 4.5% vs 2.4% and RTX 4.5% vs 8.5% (supplementary Table 3).

Comparison of pSS-CV vs HCV-CV patients

The differences between the 71 pSS-CV and the 76 HCV-CV matched patients are presented in Table 3. As expected, dry eyes (98.6% vs 21.1%, $p < 0.0001$, OR = 262.5, 95% CI: 42.14–2680), dry mouth (97.2% vs 18.4%, $p < 0.001$, OR = 152.8, 95% CI: 34.41–657.8) and salivary gland enlargement (53.6% vs 0%, $p < 0.0001$) were more frequent among pSS-CV patients compared to HCV-CV. In addition, lymphadenopathy (31% vs 4%, $p < 0.001$, OR = 10.78, 95% CI: 3.29–35.17), arthritis (25.7% vs 10.8%, $p = 0.035$, OR = 2.85, 95% CI: 1.17–6.86), Raynaud's phenomenon (47.9% vs 22.4%, $p = 0.002$, OR = 3.18, 95% CI: 1.59–6.45), interstitial renal disease (10% vs 1.4%, $p = 0.030$, OR = 8.11, 95% CI: 1.36–92.47), hypergammaglobulinemia (69.7% vs 26.2%, $p < 0.001$, OR = 6.49, 95% CI: 2.98–13.41) and type II IgMκ cryoglobulinemia (97% vs 80.3%, $p = 0.035$, OR = 7.86, 95% CI: 1.15–85.55) were more often observed in pSS-CV compared to HCV-CV patients. On the contrary, HCV infection was associated with increased frequency of fatigue (98.6% vs 59.2%, $p < 0.0001$, OR = 47.64, 95% CI: 8.18–495.9), peripheral nervous system vasculitic involvement (71.6% vs 25.4%, $p < 0.0001$, OR = 7.43, 95% CI: 3.59–15.57) and low C4 (98.5% vs 88.6%, $p = 0.033$, OR = 8.51, 95% CI: 1.22–95.82), compared to pSS. The prevalence of lymphoma development was significantly higher in pSS-CV compared to HCV-CV patients (47.9% vs 13%, $p < 0.0001$, OR = 6.12, 95% CI: 2.7–14.4). After Bonferroni correction, arthritis, Raynaud's phenomenon, interstitial renal disease, low C4 and type II cryoglobulinemia were found with no statistically significant difference between the 2 groups (Table 3).

Discussion

Cryoglobulinemic vasculitis is a rare disease presenting in approximately 4–11% of pSS patients [16,22,23]. Despite the rarity, the disease exhibits two major clinical elements leading to poor outcome in pSS, which are systemic vasculitis and lymphoma development. This information has been concluded by several case and cohort studies in the past [4,16,23–25]. However, the major determining factor of those studies was the presence of cryoglobulinemia while more

Table 3
Comparison of the clinical, laboratory and histologic features between pSS-CV and HCV-SS patients.

	pSS-CV	HCV-CV	*P*-value	*P*-value*
Number of patients	71	76		
Gender (female)% (n)	97.2 (69/71)	97.4 (74/76)	1	1
Median age (years)	50 (range: 21–75)	51 (range: 29–62)	0.718	1
Clinical Features% (n)				
Dry mouth	97.2 (69/71)	18.4 (14/76)	<0.0001	<0.0001
Dry eyes	98.6 (70/71)	21.1 (16/76)	<0.0001	<0.0001
SGE	53.6 (37/69)	0(0/75)	<0.0001	<0.001
Lymphadenopathy	31 (22/71)	4(3/75)	<0.0001	<0.001
Fatigue	59.2 (42/71)	98.6 (69/70)	<0.0001	<0.0001
Arthralgia/myalgia	71.8 (51/71)	73.7 (56/76)	0.797	1
Arthritis	25.7 (18/70)	10.8 (8/74)	0.035	1
Myositis	0(0/71)	0(0/75)	1	1
Raynaud's phenomenon	47.9 (34/71)	22.4 (17/76)	0.002	0.062
Purpura	90.1 (64/71)	88.2 (67/76)	0.902	1
Vasculitic ulcer	12.7 (9/71)	25(19/76)	0.090	1
PNS	25.4 (18/71)	71.6 (53/74)	<0.0001	<0.0001
CNS	2.8 (2/71)	1.4 (1/71)	1	1
Liver				
Sclerosing Cholangitis	0(0/71)	0(0/76)	1	1
AIH	1.4 (1/71)	0(0/70)	1	1
PBC	1.4 (1/71)	0(0/72)	0.496	1
Lung				
ILD	8.6 (6/70)	1.3 (1/75)	0.056	1
Small airways disease	13 (7/54)	4(3/75)	0.093	1
Kidney				
Interstitial renal disease	10 (7/70)	1.4 (1/74)	0.030	0.93
Glomerulonephritis	11.4 (8/70)	23.7 (18/76)	0.085	1
Splenomegaly	2.8 (2/71)	10.8 (8/74)	0.097	1
Lymphoma	47.9 (34/71)	13(9/69)	<0.0001	<0.001
Biological features% (n)				
RF	95.7 (67/70)	87.1 (61/70)	0.128	1
Low C4	88.6 (62/70)	98.5 (66/67)	0.033	1
Cryoglobulinemia type II	97 (32/33)	80.3 (61/76)	0.035	1
Hypergammaglobulinemia	69.7 (46/66)	26.2 (17/65)	<0.0001	<0.0001
Monoclonal gammopathy	45.5 (30/66)	55.5 (20/36)	0.442	1
Leukopenia	17.4 (12/69)	14.7 (10/68)	0.845	1
Thrombocytopenia	3(2/67)	11.4 (8/70)	0.097	1

SGE: salivary gland enlargement, PNS: peripheral nervous system, CNS: central nervous system, AIH: autoimmune hepatitis, PBC: primary biliary cirrhosis, ILD: interstitial lung disease, RF: rheumatoid factor.
*: Bonferroni adjusted p-value.

precise data according to the international and validated classification criteria for CV are limited [9,10,14,16,26,27]. Thus, the clinical presentation of pSS-CV, as well as the characteristics that rule the outcome and the definition of the clinical phenotype of pSS-CV are still unmet needs.

The present study was conducted to address the clinical presentation of CV in unselected patients with pSS. The tools that were used include: a) an integrated Greek-Italian population, from 5 clinical centers evaluated by physicians, highly experienced with pSS-CV, b) application of unified and validated criteria for both pSS and CV, c) carefully selected triple matched 1:2 controls from the same clinical center, and d) analysis of the results by applying not only the classic statistics, but beyond that, a data driven approach with an unbiased selection of variables, to point out features associated with CV which are involved in lymphoma development. HCV infection often mimics pSS, sharing common clinical manifestations, including sicca symptoms and CV. To address this challenging diagnostic question, we compared the clinical picture of pSS-CV with that of HCV-CV.

To our knowledge this is the largest study of pSS-CV patients fulfilling the 2011 CV classification criteria. The prevalence of cryoglobulins is within the range reported by previous studies, [7–9,14,16] corresponding to 10% of the evaluated pSS patients. The clinical picture described in this report is in line with previous studies, but some points are presented for the first time and deserve special attention: a) approximately 60% of pSS-CV patients had their first CV manifestation within the first year from pSS onset, b) pSS-CV is heralded by non-specific clinical manifestations, such as arthralgia/myalgia, arthritis or Raynaud's phenomenon, c) high prevalence of skin vasculitis, extending from the one third of patients at disease onset to almost all patients after many years of follow up and d) a time-related pattern for the appearance of glomerulonephritis and peripheral neuropathy was not observed. One-third of the associated NHL cases occurred during the first 5 years after CV onset, but the diagnosis of NHL for the majority of pSS-CV patients, spread out in 20 years, since CV diagnosis. Based on these observations, since pSS-CV is usually heralded by non-specific manifestations of pSS, it is strongly recommended that, pSS patients must be evaluated properly at pSS onset for the presence of cryoglobulins. Following early diagnosis, pSS-CV patients should undergo a close follow up for many years, since internal organ vasculitis or lymphoma can very well be late sequels. These findings can also start a discussion, whether early intervention with targeted B-cell treatments, might be instituted after the detection of cryoglobulins, even if this is associated with non-specific symptoms, in an attempt to prevent overt vasculitis and/or lymphoma. Finally, the present study has clearly shown that pSS-CV and HCV-CV, are distinct entities, since pSS-CV patients present much more frequently with sicca manifestations, lymphadenopathy, arthritis and lymphoma, mainly in the context of type II cryoglobulinemia with an IgMκ mRF [28].

In accordance with previous reports [16,26,29], purpura is the most prevalent CV-specific manifestation of pSS-CV. This study, adds that purpura is also the most common CV-specific presenting

manifestation occurring in one-third of patients within the first year after pSS onset, thus explaining why purpura at pSS diagnosis is an excellent predictor for future lymphoma development [30]. Although the clinical expression of pSS-CV is spreading across time, this study showed a temporal clustering of CV and pSS onset in 60% of pSS-CV patients. In line with this, one third of pSS-CV will eventually develop a non-Hodgkin lymphoma of B cell origin within the first 5 years of pSS onset. Taken together, it appears that this subset of pSS patients possess a discrete B cell monoclonal population, producing an IgMκ monoclonal RF, very early during pSS disease course. Importantly, previous data point out that the monoclonal component is composed within the salivary glands [31,32], explaining why salivary gland enlargement in several reports is an independent predictor of future lymphoma development. The production and perpetuation of mRFs within the inflamed salivary glands are probably attributed to the formation of ectopic germinal center like structures (EGCS), which are more frequently found in pSS-CV patients compared to cryoglobulin negative controls. On the contrary, in HCV-related cryoglobulinemia [12], B-cell clonal expansion is mainly localized within the bone marrow and the liver [33]. Compared to HCV-CV, pSS-CV patients carry an increased risk of lymphoma that could be explained by the occurrence of MALT lymphomas unrelated to cryoglobulinemia [12] and by the more intense and persistent autoreactive B cell activation as attested by the higher frequency of lymphadenopathy, hypergammaglobulinemia and specific autoantibodies, observed in pSS-CV patients. On the other hand, HCV-CV patients had a remarkably increased peripheral nervous system involvement which could be attributed to 2 distinct underlying mechanisms: a) inflammation of the vasa nervorum not only due to cryoglobulins but also to anti-HCV/HCV immune complex deposition, and b) direct HCV mediated inflammation of the nerves [34–36].

Previous studies have clearly shown that cryoglobulinemia is associated with increased morbidity and mortality, serving also as one of the strongest laboratory predictors for future lymphoma development, either with or without CV [13,30,37]. The answer to the question whether cryoglobulinemia or CV serves better as lymphoma risk factor is still unaddressed. Indeed, previous studies, using small number of patients, showed that neither CV nor cryoglobulinemia were proven risk factors for lymphoma in the multivariate model, although CV was correlated with increased mortality [16]. In our dataset, we performed a data driven analysis to identify lymphoma associated features including both cryoglobulinemia and the presence of CV. Although, cryoglobulinemia was emerged as an independent feature, this finding is random, since in our specific dataset both cryoglobulinemia and CV possess the same power of significance, according to the FCBC algorithm and, eventually one of them, but not both could be selected as a potentially independent variable. The FCBF algorithm categorizes on a mathematical based manner, features and variables with minimal inter-correlation and therefore it was unexpected to pre-select both cryoglobulinemia and CV as potentially independent variables for the logistic regression (LR) model. Thus, it was highly unlikely to compare cryoglobulinemia and CV as independent variables for any type of LR model, since the first variable is a prerequisite for the second. Finally, to address this question, we analyzed concomitantly the group of the 44 pSS cryoglobulin positive patients without vasculitic tissue damage and compared it with the 71 pSS CV patients. Around half of the pSS-CV patients had NHL, while that was true only for one fourth of cryoglobulin positive CV negative pSS patients. In the latter group the determining factor for lymphoma development was the presence of mRF in the cryoprecipitate (87.5% of patients with lymphoma) i.e. type II cryoglobulinemia and not type III. In the pSS-CV patients where the prevalence of lymphoma was double, the presence of IgMκ mRF type II cryoglobulinemia was 97%. At this point, we feel it is important to emphasize that the capacity of serum cryoglobulins to precipitate in tissues may be affected by many factors: a) the quantity of circulating cryoglobulins as a net balance between production and clearance [38,39], b) the degree of the affinity-avidity of the monoclonal component with rheumatoid factor activity against the polyclonal IgG component [40], c) the physicochemical properties of cryoglobulins such as sialylation of the Fc portion [41] and d) several environmental factors including temperature, pH and the presence of plasma hyperfiltration conditions in specific tissues [40,42]. Thus, the cryoprecipitable IgMκ mRF, the common denominator between the two groups, operates as either a double or single sword edge in patients with pSS-CV and pSS with cryoglobulinemia only, regarding lymphoma development. In the second case, it represents only the B-cell clonal expansion, whilst in the first case it is the major element responsible for the generation of the complex disease of CV that can be seen as a composite index, born by nature. This can explain why in several previous studies, many items of CV served as predictive factors for future lymphoma development. Another issue that remains inadequately addressed in the literature, is the net effect of specific treatment modalities on lymphoma development. In this series of pSS-CV patients, the majority of those with severe vasculitic involvement were treated with corticosteroids, hydroxychloroquine, B cell depletion therapy (rituximab) and plasmapheresis. In very few cases cyclophosphamide was administered, while methotrexate (MTX) and azathioprine (AZA) were more commonly used in pSS-CV patients compared to cryoglobulin negative controls. pSS-CV patients without lymphoma have been also treated more frequently with MTX and AZA compared to those with lymphoma. Therefore, it seems that the additional risk from the excess use of MTX and AZA among pSS-CV patients compared to controls is low, suggesting that systemic immunosuppression confers low risk to lymphoma development.

In this work, we present our results with and without Bonferroni correction that represents one of the most widely used methods for p-values adjustment to avoid type I error in the context of multiple comparisons. However, it has been supported that Bonferroni correction is very stringent and augments the occurrence of type II error [43]. Therefore, many researchers including the authors of this manuscript, choose not to make any adjustments for multiple comparisons and present data on the original form [43], while part of the scientific community believes that Bonferroni correction is of limited use in biomedical science [44]. For all reasons mentioned above, we show the original and Bonferroni adjusted p-values, adopting in this way all scientific opinions. At the end, CV was proven a major risk factor for lymphoma development. The statistical significance of this element was not lost after Bonferroni correction, a finding that further strengthens our conclusions. On the other hand, the non-specific features of CV such as Raynaud's phenomenon and fatigue did not retain statistical significance after adjustment, implying that a type II error may have occurred.

The current study has some limitations. Even if the group of pSS-CV patients is the largest ever described, its number is still relatively small and therefore, a larger group would allow a better power analysis. While our study was amply powered, the resulting confidence intervals of effect estimates, support the necessity for the refinement of exact effect size estimates, in further prospective investigations. The fact that not all pSS patients included in the total study population have been evaluated for cryoglobulins, probably underestimates the real prevalence of both cryoglobulinemia and CV, pointing out a selection bias. However, the evaluated pSS patients represent more serious cases and therefore, our results can be generalized, as they reflect the real clinical practice. In addition, the inclusion of non-specific manifestations in the 2011 classification criteria of CV is an inner deficit, leading to overestimation of such manifestations in the context of pSS CV, since they can be attributed to either the CV or pSS itself. Such limitations, regarding not only the clinical phenotyping of CV in pSS but also the need for validation, are expected to be overcome through a large multicenter study population in the context of the HarmonicSS project. Another limitation is the heterogeneity in

terms of ethnicity, genetic background and environmental influence between Greek and Italian pSS patients who participated in the study. Finally, the FCBF/LR data driven analysis was applied on the dataset of pSS-CV and cryoglobulin negative patients, of whom 46 had lymphoma, a relatively small number that is anticipated to affect the sensitivity level of the model.

In summary, CV associated with pSS constitutes a specific clinical phenotype of pSS, associated with both an inflammatory component, clinically expressed as vasculitis and NHL development. The presence and type of cryoglobulins should be evaluated early and during follow up in every patient with pSS. In the majority of patients, CV is appeared early after pSS onset, with non-specific clinical manifestations or purpura. The major determining factor of CV is the IgMκ mRF of type II mixed cryoglobulinemia, offering the opportunity to use B-cell targeted treatments that have the potential to halt the progress of the disease. Finally, the clinical expression of pSS-CV, has certain differences compared to HCV related CV, reflecting biologic differences which may guide the physicians in the differential diagnosis and the proper therapeutic interventions.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.semarthrit.2020.07.013.

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Occurrence and Antigenic Specificity of Perinuclear Anti-Neutrophil Cytoplasmic Antibodies (P-ANCA) in Systemic Autoimmune Diseases

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Abstract: Perinuclear anti-neutrophilic cytoplasmic antibodies (P-ANCA) recognize heterogeneous antigens, including myeloperoxidase (MPO), lactoferrin, elastase, cathepsin-G and bactericidal/permeability-increasing protein. Although P-ANCA have diagnostic utility in vasculitides, they may also be found in patients with various other systemic autoimmune rheumatic diseases (SARDs). Nevertheless, the clinical significance and the targets recognized by P-ANCA in such patients remain unclear. For this purpose, herein we investigated the occurrence of ANCA-related antigenic specificities in 82 P-ANCA-positive sera by multiplex ELISA, as well as their association with other autoantibodies. The P-ANCA-positive sera corresponded to patients with vasculitides (n = 24), systemic lupus erythematosus (n = 28), antiphospholipid syndrome (n = 5), Sjögren's syndrome (n = 7), rheumatoid arthritis (n = 3), systemic scleroderma (n = 1), sarcoidosis (n = 1) and Hashimoto's thyroiditis (n = 13). In most P-ANCA-positive patients studied (51/82, 62.3%), these autoantibodies occurred in high titers (>1:160). The analysis of P-ANCA-positive sera revealed reactivity to MPO in only 50% of patients with vasculitides, whereas it was infrequent in the other disease groups studied. Reactivity to other P-ANCA-related autoantigens was also rarely detected. Our findings support that high P-ANCA titers occur in SARD. The P-ANCA-positive staining pattern is associated with MPO specificity in vasculitides, while in other autoimmune diseases, it mostly involves unknown autoantigens.

Keywords: P-ANCA autoantibodies; myeloperoxidase; elastase; vasculitis; systemic autoimmune rheumatic diseases

1. Introduction

Anti-neutrophil cytoplasmic antibodies (ANCA) are autoantibodies, mainly of IgG isotype, directed against proteins in the cytoplasmic granules of neutrophils and lysosomal proteins of monocytes. Depending on their staining pattern on alcohol-fixed neutrophils, ANCA are classified as diffuse cytoplasmic (C-ANCA), perinuclear (P-ANCA) and atypical (A-ANCA), the first two being highly significant for the diagnosis of ANCA-associated vasculitides. Myeloperoxidase (MPO) represents the major autoantigen recognized by P-ANCA, followed by neutrophil elastase, lactoferrin, cathepsin G, bactericidal/permeability-increasing protein (BPI), catalase and lysozyme, among others [1]. C-ANCA targeting

proteinase-3 (PR3) has been associated with granulomatosis with polyangiitis (GPA), whereas P-ANCA targeting MPO is associated with microscopic polyangiitis (MPA). Patients with vasculitis and P-ANCA targeting MPO are most likely suffering from MPA (55–65%), followed by eosinophilic granulomatosis with polyangiitis (EGPA) (30–40%) and GPA (20–30%) [2]. Emerging evidence suggests that ANCA specificity associates with disease activity and may affect the clinical phenotype, as well as response to treatment, risk of relapse and long-term prognosis. To this end, MPA patients with MPO-ANCAs are more likely to develop isolated crescentic glomerulonephritis [3,4], pulmonary fibrosis and peripheral neuropathy [5,6], while MPO+-GPA patients have more frequently limited disease, without severe organ involvement, less need for cyclophosphamide or rituximab therapy and fewer relapses than those with proteinase-3 (PR3)-ANCA [7,8]. Interestingly, reappearance of MPO-ANCAs indicates relapse in more than 75% of patients [9].

Beyond MPA, P-ANCA have been described in a variety of other systemic autoimmune rheumatic diseases (SARDs), as well as chronic infections [10]. Indeed, MPO-ANCAs have been reported in systemic lupus erythematosus (SLE; 9.3%) [11], rheumatoid arthritis (RA; 4–18%) [12], Sjögren's syndrome (SS; <3%) [13] and systemic sclerosis (SScl; <2.4%) [14]. Their presence has been associated with vasculitic patterns of glomerulonephritis and/or pulmonary involvement, while other P-ANCA-specific autoantigens, such as lactoferrin, neutrophil elastase, cathepsin and lysozyme, have also been described, although without known clinical significance [13,15,16]. In this context, P-ANCA and their distinct targets may have a potential role in distinguishing clinical phenotypes, disease prognosis and/or treatment monitoring. The aim of this study was to investigate the occurrence and the autoantigenic targets recognized by P-ANCA in various SARDs.

2. Materials and Methods

2.1. Patients' Characteristics

The sera that have been examined for ANCA positivity by indirect immunofluorescence (IIF) in two highly experienced Greek diagnostic immunology laboratories (Department of Pathophysiology, School of Medicine, National and Kapodistrian University of Athens—a laboratory participating in the annual European Consensus Finding Study (ECFS) for Autoantibodies in Rheumatic Diseases in the context of EULAR and the Department of Immunology and Histocompatibility, Evangelismos General Hospital, Athens, Greece) during the past two years have been included in the study. From a total of 550 patients who were evaluated, 82 were found to be positive for the presence of P-ANCA by IIF and were included in the study. The medical records of all P-ANCA(+) patients were retrospectively analyzed and cumulative clinical, laboratory and autoantibody profile data were collected. Patients were classified into various systemic autoimmune diseases based on international classification criteria [15,17–27]. Subgroup analysis to identify clinical associations with P-ANCA autoantibodies was performed in terms of P-ANCA titers, type of autoimmune disease and comparison with control patients whenever applicable. The study was approved by the Ethics Committee of School of Medicine, National and Kapodistrian University of Athens, Greece (protocol no: 1718016656), following the general data protection regulations (GDPR) of European Union and the Helsinki Declaration principles. All sera samples were stored at −20 °C immediately after sampling and kept there until use.

2.2. Detection of P-ANCA Specificity and Associated Antigen Reactivity in Serum

The presence and titer of P-ANCAs were evaluated by standard IIF analysis on alcohol-fixed neutrophils using the NOVA Lite ANCA kit, Inova Diagnostics Inc. (San Diego, CA, USA) according to the manufacturer's instructions, followed by evaluation of the staining pattern by fluorescence microscopy. Positive sera at a dilution of 1:20 (positive cut-off threshold) were serially diluted until becoming negative and the last positive dilution was considered as the P-ANCA titer. The antigens recognized by P-ANCA were further evaluated by a commercially available multiplex ELISA (ANCA profile ELISA,

Euroimmun, Lubeck, Germany), analyzing the reactivity against MPO, lactoferrin, neutrophil elastase, cathepsin G and BPI antigens, semi-quantitatively and according to the manufacturer's instructions.

2.3. Detection of Anti-Nuclear (ANA) and Other Autoantibodies in Serum

Similar to ANCA detection, the presence and titer of ANA were evaluated by IIF using the NOVA Lite HEp-2 ANA kit, Inova Diagnostics Inc in serial serum dilutions starting from 1:160 dilution and fluorescence microscopy. Anti-Ro(SSA), anti-La(SSB), anti-Sm, anti-U1RNP, anti-Scl70 and anti-RibP(IgG) autoantibodies were tested by immunoblotting using the Euroline Anti-ENA ProfilePlus1 (IgG) and Euroline ANA Profile-3 kits, Euroimmun. The levels of IgG and IgM antibodies against cardiolipin (aCL), β2GPI and double-stranded DNA (ds-DNA) were determined by home-made ELISAs, as previously described [28–31]. Anti-CCP, anti-TPO and anti-Tg were measured by commercially available ELISAs (QUANTA Lite CCP3.1 IgG/IgA, QUANTA Lite TPO and QUANTA Lite Thyroid T ELISA kits, Inova Diagnostics Inc.) according to the manufacturer's instructions, whereas rheumatoid factor (RF) was detected by agglutination assay using commercially available latex-based reagents (RapiTex RF, Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany) according to manufacturer's instructions.

2.4. Statistics

Comparisons of categorical data were performed by chi square or Fisher exact test when cell counts were <5. For continuous variables, the Shapiro–Wilk normality test was performed initially, followed by the Mann–Whitney–Wilcoxon or *t*-test accordingly. Statistical analyses were performed using Python 3.6, and R software 4.0.3.

3. Results

3.1. Patients' Characteristics

Eighty two of 550 tested patients were found to be positive for P-ANCA autoantibodies (in titer ≥1:20 dilution), 69 (84.2%) of whom fulfilled the criteria of a systemic autoimmune rheumatic disease (SARD) and 13 (15.9%) who presented with Hashimoto thyroiditis (HT). The 69 P-ANCA-positive SARD patients included 28 (40.6%) with systemic lupus erythematosus (SLE), 24 (34.8%) with a form of systemic vasculitis (18 with microscopic polyangiitis [MPA], two each with Behcet's disease [BD] and Henoch–Schönlein purpura [HSP] and one each with aortitis and cryoglobulinemic vasculitis), 7 (10.2%) with Sjögren's syndrome (SS), 5 (7.2%) with primary antiphospholipid syndrome (APS), 3 (4.4%) with rheumatoid arthritis (RA), and one each with systemic sclerosis (SSCL) and sarcoidosis.

The majority of SARD patients (57/69, 82.6%), as well as HT patients (11/13, 84.6%) were women. The median age at the time of P-ANCA measurement was 58 years (range: 20–85) for the SARD group and 55 years (range: 34–77) for those with HT patients. A more detailed description of patients' characteristics per disease group is presented in Supplementary Tables S1 and S2.

3.2. P-ANCA Titers and Serum Autoantigen Specificity Per Autoimmune Disease

As detected by IIF, the titers of P-ANCA autoantibodies in the 82 P-ANCA-positive sera ranged from 1:20 to 1:640 (median: 640, Table 1). The majority of patients with SARD who were studied (50/69, 72.5%) presented with high P-ANCA-titer, namely, ≥1:80 (in 50/69, 72.5%) or ≥1:160 (in 43/69, 62.3%). Microscopic polyangiitis patients had higher P-ANCA titers compared to SLE patients (Figure 1). Among the 18 sera of P-ANCA-positive MPA patients, 11 (61.1%) presented reactivity to MPO (MPA-P-ANCA-MPO-positive), whereas the remaining seven did not exhibit any reactivity against the various autoantigens examined (MPA-P-ANCA-NS). On the other hand, the vast majority of the patients with other SARD were not found to recognize any of the P-ANCA-related antigens under investigation, including 25/28 (89.3%) of SLE patients studied. In fact, monospecific P-ANCA-positive patient cases with anti-MPO reactivity included one each

with SLE, APS, RA and systemic sclerosis, whereas one patient with SS reacted with elastase and a SLE patient with lactoferrin. In addition, a SLE patient had double specificity for MPO/lactoferrin (Table 2).

Table 1. Serum P-ANCA titers in the various P-ANCA-positive autoimmune disease patients studied.

Patient Groups		P-ANCA Serum Titers (No Positive)					
		≥1:640	1:320	1:160	1:80	1:40	1:20
Vasculitides	MPA (n = 18)	9	5	1	1	1	1
	BD (n = 2)	1	1	0	0	0	0
	Aortitis (n = 1)	1	0	0	0	0	0
	HSP (n = 2)	0	0	1	1	0	0
	CV (n = 1)	0	1	0	0	0	0
SLE (n = 28)		9	6	3	7	23	0
APS (n = 5)		1	1	1	1	0	1
SS (n = 7)		5	0	0	0	0	2
RA (n = 3)		2	0	0	1	0	0
SSCL (n = 1)		0	1	0	0	0	0
Sarcoidosis	(n = 1)	0	0	1	0	0	0
Hashimoto	(n = 13)	2	1	4	2	1	3

P-ANCA: perinuclear antineutrophil cytoplasmic antibodies, MPA: microscopic polyangiitis, BD: Behcet's disease, HSP: Henoch–Schonlein purpura, CV: cryoglobulinemic vasculitis, SLE: systemic lupus erythematosus, APS: antiphospholipid syndrome, SS: Sjögren's syndrome, RA: rheumatoid arthritis, SSCL: systemic sclerosis.

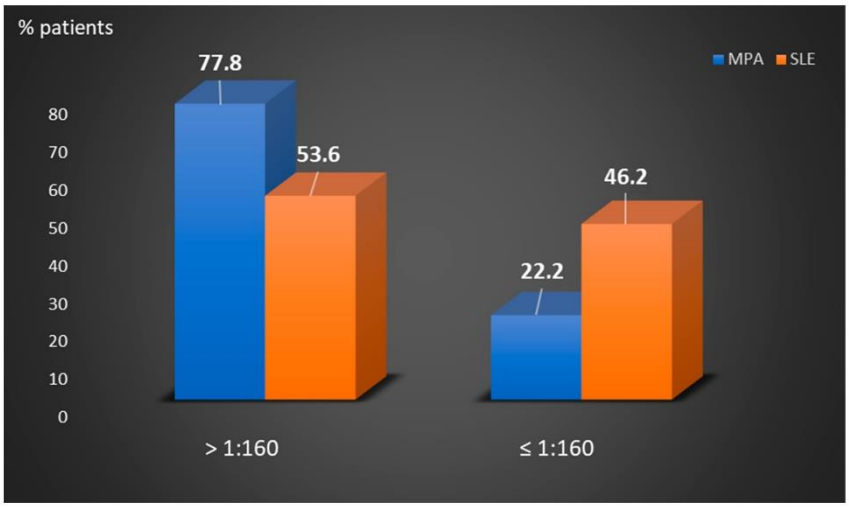

Figure 1. Comparison of the percentages of P-ANCA-positive MPA and SLE patients according to low ≤1/160 or high >1/160 titers. MPA: microscopic polyangiitis, SLE: systemic lupus erythematosus.

Table 2. P-ANCA-related antigenic specificities in the various P-ANCA-positive autoimmune disease patients studied.

Autoimmune Diseases		Antigens Recognized by P-ANCA Positive Sera (No Positive)					
		MPO	Elastase	Cathepsin G	BPI	Lactoferrin	MPO/ Lactoferrin
Vasculitides	MPA (n = 18)	**11**	0	0	0	0	0
	BD (n = 2)	**1**	0	0	0	0	0
	Aortitis (n = 1)	0	0	0	0	0	0
	HSP (n = 2)	0	0	0	0	0	0
	CV (n = 1)	0	0	0	0	**1/1 (100)**	0
SLE (n = 28)		**1**	0	0	0	**1/28 (3.6)**	**1/28 (3.6)**
APS (n = 5)		**1**	0	0	0	0	0
SS (n = 7)		0	**1**	0	0	0	0
RA (n = 3)		**1**	0	0	0	0	0
SSCL (n = 2)		**1**	0	0	0	0	0
Sarcoidosis (n = 1)		0	0	0	0	0	0
Hashimoto (n = 13)		0	0	0	0	0	0

P-ANCA: perinuclear antineutrophil cytoplasmic antibodies, MPO: myeloperoxidase, BPI: bactericidal/permeability-increasing protein, MPA: microscopic polyangiitis, BD: Behcet's disease, HSP: Henoch–Schonlein purpura, CV: cryoglobulinemic vasculitis, SLE: systemic lupus erythematosus, APS: antiphospholipid syndrome, SS: Sjögren's syndrome, RA: rheumatoid arthritis, SSCL: systemic sclerosis. Positive values are highlighted by bold type letters.

3.3. Autoantibody Profile of P-ANCA Positive Patients and P-ANCA Related Specificity

Microscopic polyangiitis-P-ANCA patients had increased frequency of ANA with a titer ranging from 1:160 to 1:1280 [1:160: 30%, n = 3/10, 1:320: 50%, n = 5/10, 1:640: 10%, n = 1/10 and 1:1280: 10%, n = 1/10) and RF (27.8%, n = 5/18) (Figure 2A). Compared to MPA-P-ANCA-NS, MPA-P-ANCA-MPO-positive patients had higher prevalence of ANA (63.6%, n = 7/11 vs. 42.9%, n = 3/7) and RF (36.4%, n = 4/11 vs. 14.3%, n = 1/7) and lower prevalence of anti-β2GPI-IgM (0% vs. 14.3%, n = 1/7), anti-Ro52/Ro60 (0% vs. 14.3%, n = 1/7) and anti-TPO (9,1%, n = 1/11 vs. 14.3%, n = 1/7), although these differences did not reach statistical significance (data not shown). The autoantibody profile of P-ANCA-positive SLE patients is presented in Figure 2B. All were ANA positive (28/28), with the vast majority having anti-dsDNA antibodies (in 21/25, 75%) and anti-Ro60/SSA autoantibodies (in 14/28, 50%). The majority of P-ANCA-positive SS patients were ANA positive (5/7, 71.4%) and approximately half of them had also anti-Ro52/Ro60 autoantibodies (3/7, 42.9%). A detailed description of the autoantibody profile of the remaining SARD, as well as HT patients, is presented in Supplementary Table S3.

3.4. Disease Features of P-ANCA Positive Patients

The small size of each disease group hampered the reliable statistical analysis of possible associations between P-ANCA-reactivity and clinical phenotypes. However, it seems that the clinical features of P-ANCA-positive patients fall within the typical disease clinical spectrum. More analytically, MPA-P-ANCA patients presented with non-specific clinical manifestations, including fatigue (14/18) and fever (12/18), as well as organ threatening disease, such as interstitial lung disease (ILD) and/or infiltrates (12/18) and glomerulonephritis (11/18). The most frequent clinical manifestations of SLE-P-ANCA patents were skin rash and/or photosensitivity (22/28), arthralgia/arthritis (18/28), anemia of chronic disease (13/28) and renal involvement (9/28). Furthermore, the three SLE patients recognizing P-ANCA-related antigens presented with different clinical phenotype. The anti-MPO-positive patient suffered from fatigue, fever, arthralgia/arthritis, photosensitivity and hematological findings, including anemia, leukopenia and thrombocytopenia; the anti-lactoferrin-positive SLE patient had more severe disease with non-specific manifestations (fatigue, fever, sicca symptoms), inflammatory arthritis, skin rash/photosensitivity,

lymphadenopathy, serositis, Libman-Sacks endocarditis, enteritis, anemia and leukopenia, while the SLE patient with the double anti-MPO/lactoferrin specificity presented with fatigue, sicca manifestations, arthralgias, skin rash/photosensitivity and hematological findings (anemia, leukopenia and thrombocytopenia). The SS-P-ANCA+ patients all had sicca symptoms, musculoskeletal manifestations (6/7), Raynaud's phenomenon (3/7) and anemia of chronic disease (3/7). The anti-elastase-positive SS patient had sicca manifestations, lymphadenopathy and ILD. The P-ANCA-positive APS patients had major vascular events, including pulmonary emboli, and thrombocytopenia, while the anti-MPO/elastase patient had lupus-like phenotype with mouth ulcers, alopecia, Raynaud's phenomenon, pulmonary emboli, leukopenia and thrombocytopenia. The SSCL-anti-MPO+ patient developed acute renal failure. The clinical and laboratory features of all P-ANCA(+) patients included in the study are summarized in Supplementary Tables S1 and S2.

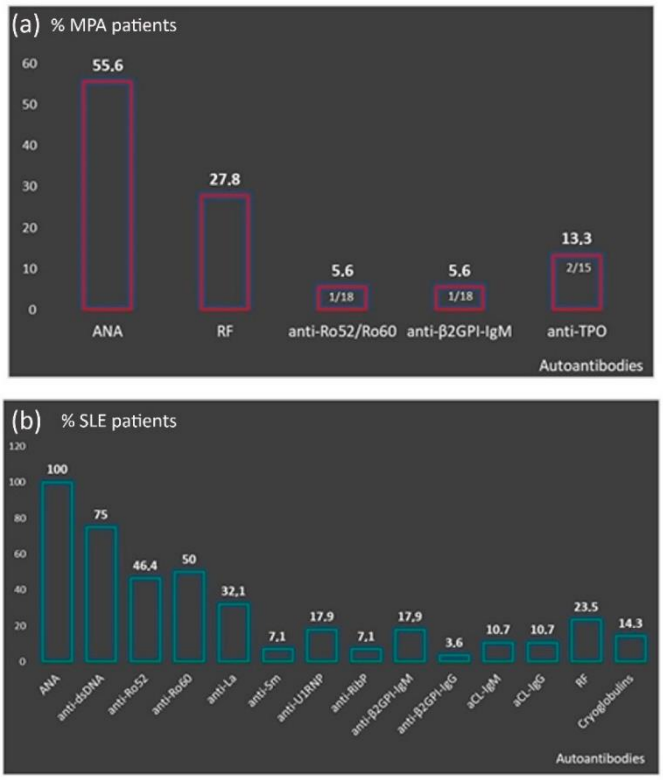

Figure 2. Autoantibody profile of P-ANCA positive patients. (**a**) Presence other than P-ANCA autoantibodies in MPA patients; (**b**) distribution of autoantibodies in the context of SLE. P-ANCA: perinuclear antineutrophil cytoplasmic antibodies, MPA: microscopic polyangiitis, SLE: systemic lupus erythematosus, ANA: antinuclear antibodies, RF: rheumatoid factor, aCL: anti-cardiolipin.

4. Discussion

In this report, we investigated the occurrence of P-ANCA autoantibodies and their specificity in various autoimmune diseases. In accordance with previous studies [2], this study further indicates that MPO is the predominant autoantigen targeted by P-ANCA in MPA patients, whereas reactivity to other P-ANCA-related autoantigens, such as lactoferrin, may only sporadically be observed [32]. Nevertheless, our study indicates that the antigenic specificity of P-ANCA autoantibodies remains elusive in a significant proportion of such patients. On the other hand, our results support that high titers of P-ANCA autoantibodies are frequently observed in patients with systemic autoimmune diseases, other than

MPA. Interestingly, despite the relatively high P-ANCA titers, the vast majority of these patient groups have unidentified specificities. In line with previous reports [4,11,12,16], the most frequently recognized antigen in P-ANCA-positive patients is MPO, whereas reactivity against other P-ANCA-related autoantigens, such as lactoferrin and elastase, were rarely observed. Importantly, P-ANCA-positive SLE and MPA patients presented with enriched autoantibody profile, implying systemic autoimmune responses against ubiquitous self-antigens. Although the number of SLE P-ANCA positive patients is small, it is noteworthy that P-ANCA positivity among these patients is associated with skin, renal and hematologic manifestations. Interestingly, the anti-MPO positive lupus patient had no renal involvement, while anti-lactoferrin specificity was linked to the most severe clinical phenotype as opposed to double anti-MPO/lactoferrin specificity relayed to a milder clinical picture. These findings imply that P-ANCA specificity may have clinical significance; however, further multicentric studies of large patient cohorts are needed to verify these observations.

Important limitations of this study were the small number of patients, as well as the small number of autoantigens included in the assay we applied. A variety of potential self-antigens may be recognized by P-ANCA autoantibodies. Thus, diverse autoantigen array and/or high throughput biotechnologies are required to reveal potentially hidden specificities of P-ANCA reactivity. On the other hand, although limited, the misinterpretation of A-ANCA, as P-ANCA, cannot be excluded since our detection was based on immunofluorescence detection of only ethanol-fixed neutrophils. The parallel use of ethanol and formaldehyde-fixed neutrophils for the detection of P-ANCA has been reported to discriminate false P-, atypical ANCA [33]. However, this approach applies mainly for recognizing P-ANCA targeting MPO, whereas it has been reported to be non-beneficial for the study of SARDs patients other than those with vasculitides [33–35]. Furthermore, the likelihood of the detection of P-ANCA-related pattern in the setting of concurrent high ANA titers cannot be excluded as ANA may produce indistinguishable immunofluorescent staining patterns on ethanol-fixed neutrophils. In fact, charge interactions between DNA and MPO may cause false positivity in MPO-ANCA in case of high anti-dsDNA sera titer [36]. However, this possibility is significantly diminished by the fact that the detection of the characteristic perinuclear ring pattern is a prerequisite finding for the diagnosis of P-ANCA staining pattern in our departments.

In summary, P-ANCA are present in sera of patients with various systemic autoimmune diseases in high titers and are associated with ANA, confirming to most likely be in support ofthe systemic nature of autoimmunity. The results of this study indicate that, although MPO represents the most common P-ANCA specificity for MPA, autoantibodies to additional novel neutrophilic self-antigens are likely present in P-ANCA-positive autoimmune disease patients. In this context, further investigation of the currently unidentified P-ANCA-related autoantigens may reveal novel and clinically useful disease markers.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cells10082128/s1, Table S1: Clinical and Laboratory features of patients with systemic vasculitis, SLE and APS, Table S2: Clinical and Laboratory features of patients with other autoimmune diseases, Table S3: Autoantibody profile of other than MPA and SLE pANCA positive patients subgrouped per disease type.

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