SAPIENZA UNIVERSITÀ DI ROMA
Department of Translational and Precision Medicine

INTERNATIONAL Ph.D. PROGRAM IN:
ARTERIAL HYPERTENSION AND VASCULAR BIOLOGY
CYCLE XXXV

CARDIOVASCULAR RISK ASSESSMENT AND DIAGNOSTIC WORK-UP IN RARE DISEASES: FOCUS ON HYPERALDOSTERONISM AND PRIMARY ANTIBODY DEFICIENCY

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Academic year: 2022/2023

PREFACE

When talking about rare diseases we immediately think about the complexity of patient enrollment, the impact of limited data and the heterogeneity of treatment protocols. All these aspects make it difficult to have a multidisciplinary approach when facing patients with rare diseases. Today, the biggest challenge in rare disease management is to implement efficient diagnostic work-up strategies that include evaluation of aspects not directly related to the original disease. Even if the first worldwide death cause are cardiovascular diseases, the nowadays cardiovascular impact evaluation in rare disease patients is not yet systematically applied. During my Ph.D. program I had the opportunity to be involved in two different projects linked by the cardiovascular aspects of rare diseases.

The first project, involving the efficacy of roxithromycin in representing a new diagnostic work-up in primary aldosteronism patients, has been coordinated by Prof. Gian Paolo Rossi with the precious scientific support of Prof. Teresa Maria Seccia.

The second project, focused on defining the cardiovascular risk profile of patients with primary antibody deficiency, has been coordinated by Prof. Marcello Rattazzi with the valued scientific support of Prof. Francesco Cinetto.

This thesis, divided in two chapters, is the result of my Ph.D. course.

Alessandro Bressan

CHAPTER 1

ANTISECRETAGOGUE EFFECT OF ROXITHROMYCIN IN KCNJ5 MUTATED ALDOSTERONE PRODUCING ADENOMA

CONTENTS

ABBREVIATIONS

APA	aldosterone-producing adenoma
AVS	adrenal vein sampling
CCB	calcium channel blocker
CT	computed tomography
DBP	diastolic blood pressure
DRC	direct renin concentration
MR	mineralocorticoid receptor
NGS	next generation sequencing
NOS	nitric oxide synthases
PA	primary aldosteronism
PAC	plasma aldosterone concentration
PCC	plasma cortisol concentration
ROS	reactive oxygen species
SBP	systolic blood pressure
XO	xanthine oxidase

ABSTRACT

Introduction: Unilateral form of primary aldosteronism (PA) is the main curable cause of endocrine hypertension cause of PA and it is in up to 66% of all cases investigated with adrenal vein sampling (AVS). Mutations in the KCNJ5 potassium channel involve up to 70% of unilateral PA. The *in vitro* evidences of macrolide antibiotics specifically inhibit the altered function of mutated KCNJ5 channels has opened new horizons for the diagnosis and treatment of unilateral PA with KCNJ5 mutations in that it can allow identification and target treatment of PA patients harbouring a mutated unilateral PA.

Aim: To test the effect of roxithromycin on aldosterone secretion and blood pressure in vivo in patients without PA, and in those with PA subtyped by AVS and examined according to the presence or absence of KCNJ5 mutation.

Methods: We enrolled consecutive hypertensive patients undergoing screening for secondary hypertension, from January 2018 to June 2022. Each patient received a single oral dose of roxithromycin and, after the diagnostic work-up, baseline values of plasma aldosterone concentration (PAC) and blood pressure were compared with post-roxithromycin values. Next-generation sequencing (NGS) was used to identify KCNJ5 mutated from wild-type forms of unilateral PA. Response to roxithromycin was compared between non-PA, non-unilateral PA and unilateral PA. In unilateral PA we focused on KCNJ5 mutated vs wild-type patients.

Results: After roxithromycin administration, patients with unilateral PA carring KCNJ5 mutation showed a decrease in PAC ($p=0.030$) that did not occur in unilateral PA KCNJ5 wild-type. In these groups systolic blood pressure (SBP) and diastolic blood pressure (DBP) did not change in response to roxithromycin. However, in non-PA group a decrease in PAC was observed ($p<0.001$) with a decrease in plasma cortisol concentration (PCC) ($p<0.001$) and systolic ($p<0.001$) but not diastolic blood pressure. After adrenalectomy, roxithromycin did not induce any change in PAC in KCNJ5 mutated unilateral PA.

Conclusion: Roxithromycin administration induces a decrease in aldosterone production in patients with KCNJ5 mutation but not in wild-type, confirming its ability *in vivo* to blunt aldosterone production by blocking the mutated Kir3.4 sodium channel without affecting wild-type forms. As

the decrease in PAC in the same patients did not occur after adrenalectomy, the roxithromycin effect is unequivocally attributable to the KCNJ5 mutated unilateral PA.

BACKGROUND

PRIMARY ALDOSTERONISM

Arterial hypertension is a major cardiovascular risk factor (Rossi, Seccia, et al.). Primary aldosteronism (PA) represents the main curable cause of human endocrine hypertension (Rossi, Bernini, et al.). It has been estimated it affects more than 11% of patients referred to specialized hypertension centers and over 20% of patients with resistant hypertension (Douma et al.). Even if PA was discovered in 1954 by Lityński (Litynski) and then independently by Jerome Conn, mechanisms involved in disease development were just recently described. PA is caused by excess aldosterone production from the adrenal gland. In the majority of cases this is attributable to a unilateral aldosterone-producing adenoma (APA) or to bilateral adrenal hyperplasia (Funder et al.). Patients with PA are at increased risk of cardiovascular disease compared with age- and blood pressure- matched patients with essential hypertension. Indeed, increased risk of stroke, myocardial infarction, atrial fibrillation and renal damage has been consistently reported among different studies of patients with PA (Monticone et al.). This is attributable to the deleterious effects of aldosterone, which promotes cardiac and vascular fibrosis and tissue damage independently of blood pressure levels (Pessina et al.). The diagnostic work-up of PA is based on hormonal tests (screening and exclusion tests), followed by subtyping (adrenal CT scanning and adrenal vein sampling [AVS]) to distinguish between unilateral and bilateral disease. Clinically, unilateral forms of PA are aldosterone producing adenoma (APA) that represent one of the major surgically curable subtypes of PA. Adrenalectomy, especially when guided by AVS, cures the hyperaldosteronism and resolves or improves arterial hypertension, with markedly better outcomes when the diagnosis is made early (Rossi, Maiolino, et al.). In the case of the bilateral forms where surgery is not indicated, the diagnosis allows a targeted drug treatment based on mineralocorticoid receptor (MR) antagonists and thus prevention of cardiovascular events (Colussi et al.). Thus, an early identification of PA followed by the diagnosis of its subtypes is of paramount importance.

Mechanisms involved in PA development were recently found to involve agonistic autoantibodies against the type 1 angiotensin receptor (Wallukat et al.), a down regulation of Twik-Acid Sensitive K channels type 2 (TASK-2) (Lenzini, Caroccia, et al.) and by two mutated isoforms (G151R and L168R) in the potassium channel Kir3.4 (KCNJ5) discovered by *Choi et al.* (Choi et al.). According to a recent large meta-analysis, KCNJ5 mutation involves between 30% and 70% of APA, representing the most

prevalent mutations in these tumors (Lenzini, Rossitto, et al.). Compare with wild-type APA, patients with KCNJ5 mutations are more often women with higher plasma aldosterone concentrations and larger tumors. These differences may explain why they develop not only more marked lateralization of aldosterone production at AVS, but also a more pronounced cardiac damage (Boulkroun et al.) (Rossi, Cesari, et al.).

KCNJ5 mutations induce a loss of the K^+ selectivity of the channel Kir3.4 (Na^+ channel). Consequently, Na^+ enters in the APA cells causing their depolarization and opening the T-type calcium channels with activation of the Na^+ Ca^{2+} exchanger (Figure 1) (Kuppusamy et al.).

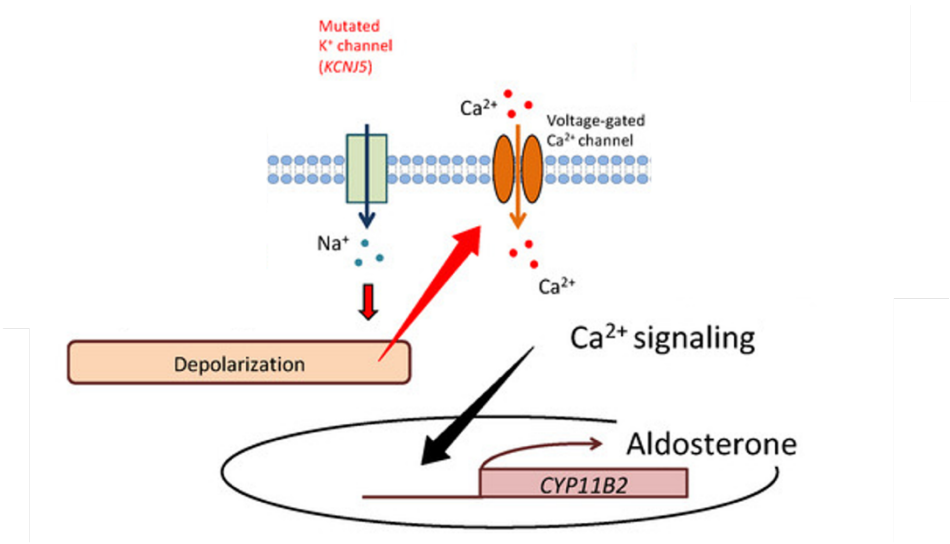

Figure 1 mutated K^+ KCNJ5 channels induce loss of selectivity of Na^+ channel leading to cell depolarization and CYP11B2 activation. From Itcho K., Biomedicines, 2021.

The increase of cytosolic Ca^{2+} activates the step-limiting enzyme aldosterone synthase (CYP11B2) leading a marked increase in plasma aldosterone concentrations. The discovery of KCNJ5 mutations in APA suggested the possibility of identifying carriers of mutated tumors simply by using circulating cell free DNA, without undertaking invasive investigations as AVS, which, according to all guidelines, represents the key test for allocating patients to adrenalectomy or life-long medical treatment.

In 2017, after undertaking a high throughput screening of over 73,000 molecules, Lifton's group identified some macrolide antibiotics and some synthesized derivatives without antibiotic activity, which specifically inhibit the altered function of mutated KCNJ5 channels (Scholl et al.). Accordingly, administration of these macrolides could specifically blunt the excessive Na^+ influx in

cells carrying mutated channel, and therefore would correct cell depolarization causing aldosterone production.

In *in vitro* experiments, those changes in aldosterone production did not occur in cells with the wild-type channel, displaying a selective effect of tested compounds (Scholl et al.).

As reported in Figure 2, the analysis of 14 macrolides and their derivatives confirmed potent effects of roxithromycin and clarithromycin, greater than erythromycin or flurithromycin.

	G151R			L168R			WT
Drug name	IC_{50} (μM)	Minimum inhibition (%)	Maximum inhibition (%)	IC_{50} (μM)	Minimum inhibition (%)	Maximum inhibition (%)	IC_{50} (μM)
Roxithromycin	0.22	9.92	105.92	0.69	3.96	81.39	No fit
Roxithromycin-D7	0.58	1.67	96.22	0.68	3.95	70.07	No fit
Idremcinal (EM574)	0.60	6.38	93.39	1.99	-1.28	66.69	No fit
Pseudo erythromycin A enol ether	0.65	1.58	11.13	No Fit	NA	NA	No fit
Clarithromycin	0.71	6.40	83.09	1.72	2.35	55.70	No fit
n-Demethyl roxithromycin	0.82	4.50	96.20	1.18	1.12	76.95	No fit
Erythromycin B	1.23	7.15	84.36	4.73	1.62	80.00	No fit
Erythromycin A oxime (roxithromycin impurity C)	2.88	2.49	84.11	8.13	0.65	60.00	No fit
Azithromycin	5.69	5.11	32.00	8.05	0.71	12.01	No fit
Anhydro-erythromycin A	8.60	5.61	60.00	No fit	NA	NA	No fit
Erythromycin C	9.19	6.72	75.00	12.18	1.41	18.43	No fit
Erythromycin	10.53	3.55	90.00	11.76	0.71	25.00	No fit
Dirithromycin	15.80	1.31	18.29	No fit	NA	NA	No fit
Flurithromycin	No fit	NA	NA	No fit	NA	NA	No fit

Figure 2 Macrolides and their derivates tested in inhibition activity of G151R and L168R isoforms in KCNJ5 mutated and wild-type HEK293 cells. From Scholl et al., JCI, 2017.

When exposed to increasing concentrations of macrolides, cells obtained from wild-type APAs showed no change in aldosterone synthase (CYP11B2) gene expression and aldosterone secretion in response to the macrolide. By contrast, both G151R and L168R APA cells exposed to clarithromycin showed a concentration-dependent decrease expression of CYP11B2, the aldosterone synthase gene (Caroccia et al.). This seminal discovery opened new horizons for the diagnosis and treatment of APA with KCNJ5 mutations. To date, however, these evidences pertain only to *in vitro* studies, and there is no proof that these molecules can lower aldosterone, and thereby correct hypertension, *in vivo* in patients with KCNJ5 mutated APA.

EFFECT OF MACROLIDES ON BLOOD PRESSURE

Macrolides are characterized by many-membered lactone ring with one or more deoxy sugars attached. Macrolides with antibiotic activity inhibit protein synthesis by binding to bacterial ribosomal RNA 23S in the desosamine sugar and the lactone ring (Dinos). By binding to different targets they induce an anti-inflammatory response and stimulate gastrointestinal motility (Schlünzen et al.).

In literature it has been reported that co-administration of macrolide antibiotic can enhance the effect of calcium channel blockers on lowering blood pressure (Westphal). This has been explained by the fact that macrolides prolong the action of calcium channel blocker by inhibiting the cytochrome P450(CYP3A4) action, which metabolise the calcium channel blockers in the liver (Fleet et al.). Some studies previous described nitric oxide synthases (NOS) activities were increased in macrolides treated animal models. Moreover, macrolides have been described to be able to decrease xanthine oxidase (XO) activity, leading to a decrease reactive oxygen species (ROS) production (Aktan et al.).

Those data suggest an overall positive effect of macrolides in endothelial function. It remains unclear if the macrolides hypotensive mediated effect may be due to a direct action on endothelial cells instead of the inhibition of calcium channel blockers (CCBs) metabolism.

MUTATION DETECTION BY NEXT GENERATION SEQUENCING

Compared to conventional Sanger sequencing using capillary electrophoresis, the short read, massively parallel sequencing technique is a fundamentally different approach that revolutionized sequencing capabilities and launched the second-generation sequencing methods, or next-generation sequencing (NGS), that provide orders of magnitude more data at much lower recurring cost. NGS based approaches directly determine the nucleic acid sequence of a given DNA or cDNA molecule.

It presents several advantages including a single-nucleotide resolution making possible to detect related genes, alternatively spliced transcripts, allelic gene variants and single nucleotide polymorphisms; a higher dynamic range of signal and less DNA/RNA as input. Moreover, NGS sequencing present higher reproducibility and priori knowledge of the genome or genomic features is not required. In addition to broadly improving the knowledge on cancer conceptions, NGS promoted the development of personalized medicine, giving one powerful instrument to understand each

patient's disease and its unique genetic features. Finally, NGS allows not only to identify the most common known alterations, but also the long tail of rare mutations that occur each in less than 1% of the patients.

AIM OF THE STUDY:

Aim of the study was to test the effect of roxithromycin on aldosterone secretion and blood pressure in vivo in patients without PA, and in those with PA subtyped by AVS and examined according to the presence or absence of KCNJ5 mutation.

To this end two different objectives has been pursued, each one targeted to verify:

Objective 1: if the change of PAC in response to mutated KCNJ5 channel occurs in KCNJ5-mutated APA.

Objective 2: if the plasma aldosterone concentration and blood pressure change in response to roxithromycin could be useful for the screening of PA with a KCNJ5-mutated APA.

METHODS:

PATIENTS AND STUDY DESIGN

This is a perspective case control study enrolling consecutive hypertensive patients undergoing screening for secondary hypertension, referring to the Clinica dell'Ipertensione Arteriosa of the University of Padova, Italy, a Center of Excellence of the European Society of Hypertension, from January 2018 to June 2022.

After a washout from confounding treatments, each patient undergoes to the screening for PA resting on bed for 60 min and collecting BP values and a blood draw. Patients than received a single oral dose of 150mg of roxithromycin (switched to 300mg in October, 4th 2019 to increase plasma roxithromycin concentration) and after 60 min BP values and a blood draw were collected again. The dose of 150mg should yield peak plasma concentration of 5-12ug/mL, which are higher than the IC50 measured in vitro (0.18-0.53 mcg/mL). The baseline values of PAC, DRC, cortisol and BP were compared to the post- roxithromycin values. In the hypokalemic patients normal serum potassium values have been achieved with oral KCL supplementation to reduce the risk of QT prolongation with the macrolides used.

After the screening procedure, patients have been divided in those with no evidence of PA and those with PA diagnosis, according to Endocrine Society Guidelines, European Society of Hypertension Consensus and High risk STOP-PA score/ML algorithms (Mulatero et al.). Patients with PA has been classified in unilateral form of PA (APA) and non-unilateral form of PA (bilateral hyperplasia), according to imaging and AVS results. Patients with APA undergoes to adrenalectomy. Biopsies obtained from APA tissues were used for the identification of KCNJ5 mutation.

Inclusion criteria were:

- A signed informed consent form
- A diagnosis of hypertension defined either as:
    - Use of antihypertensive drug (s)
    - Arterial hypertension: in untreated patients this must be confirmed by daytime ambulatory blood pressure monitoring (ABPM), or home blood pressure monitoring, with blood pressure higher or equal to 135 mmHg for systolic blood pressure and/or higher or equal to 85 mmHg for diastolic blood pressure.

Exclusion criteria were:

- history of allergy/intolerance to any macrolides;
- refusal of the patient to undergo dynamic testing;
- refusal of the patient to undergo AVS and/or contraindications to the general anesthesia that is required for laparoscopic adrenalectomy;

Informed consent has been obtained from all individual participants included in the study. All procedures performed in the study are in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This protocol was registered to Clinical Trial Registry as 4283/AO/17 and the study has been started after approval of the Ethics Committee of the University of Padua.

ROXYTHROMYCIN QUANTIFICATION IN PLASMA

To access if changes in plasma aldosterone concentration and blood pressure were related to the roxithromycin effect, plasma concentration of roxithromycin were measured by using high performance liquid chromatography (HPLC) system (Główka and Karaźniewicz-Łada). The oral dose of administered roxithromycin should yield peak plasma concentration of 5–12 mcg/mL, which are higher than the IC50 measured in vitro (0.18 – 0.53 mcg/mL) for inhibition of aldosterone production (Maiolino et al.).

KCNJ5 MUTATION DETECTION

Mutation in KCNJ5 were detected by using NGS sequencing. DNA for NGS sequencing has been obtained by tissue biopsy or buffy-coat and extracted by using DNeasy Blood & Tissue kit (Quiagen™). DNA obtained has been quantified by using Qubit 4 Fluorometer (Thermo Fisher Scientific™). Libraries has been prepared with xGENTM DNA Library Prep EZ kit (Integrated DNA Technologies, IDT). For NGS sequencing of the selected DNA libraries, Illumina's MiSeq instrument was used following the protocol "MiSeq System, Denature and Dilute Libraries Guide - Protocol A: Standard Normalization Method" (Illumina™). After appropriate dilutions, the final concentration of the library was 12pM. The flow cell used was MiSeq Reagent Micro Kit v2 (300 cycles, Illumina™).

Bioinformatic analysis of the data obtained at the end of sequencing was carried out using two different bioinformatic software: BaseSpace, developed by Illumina™, and CLC Genomics, developed by Qiagen™. The use of these bioinformatics software using different algorithms allows the data to be compared and verified, resulting in a more reliable overall result.

STATISTICAL ANALYSIS

Statistical analysis was performed using SPS Statistics version 28.0.1 (IBM™ New York, US). Continuous variable data were expressed as mean ± standard deviation if normally distributed or median ⍰ standard deviation if not. Categorical data were expressed like number of subjects and percentage of cases on present data. Comparisons between groups of continuous variables were assessed using a parametric ANOVA or a non-parametric Kruskal–Wallis test, according to the results of the Shapiro–Wilk test of normality. Fisher exact test was used to compare two categorical variables, Pearson's chi square test was used to compare more than two categorical variables.
Within-patient comparison of paired t-samples test has been used in the whole cohort and in the APA and non-APA sub-cohorts; Pearson's χ^2 test was used for categorical variables. p value less than 0.05 was considered significant.

RESULTS

A total of 425 hypertensive patients were included in the study (Figure 3). 341 patients showed no evidence of PA while 78 showed PA. However, 6 patients did not complete the diagnostic work-up before the data analysis. Therefore, they had to be excluded from the study. After AVS of PA patients 59 had a unilateral form while 19 a non-unilateral form. Following guidelines for treatment of unilateral form of PA, 49 patients undergo to adrenalectomy while 10 patients are waiting for surgery. NGS sequencing was performed in 44 excised adrenals and showed KCNJ5 mutation in 20 patients and no mutation in 24 wild type.

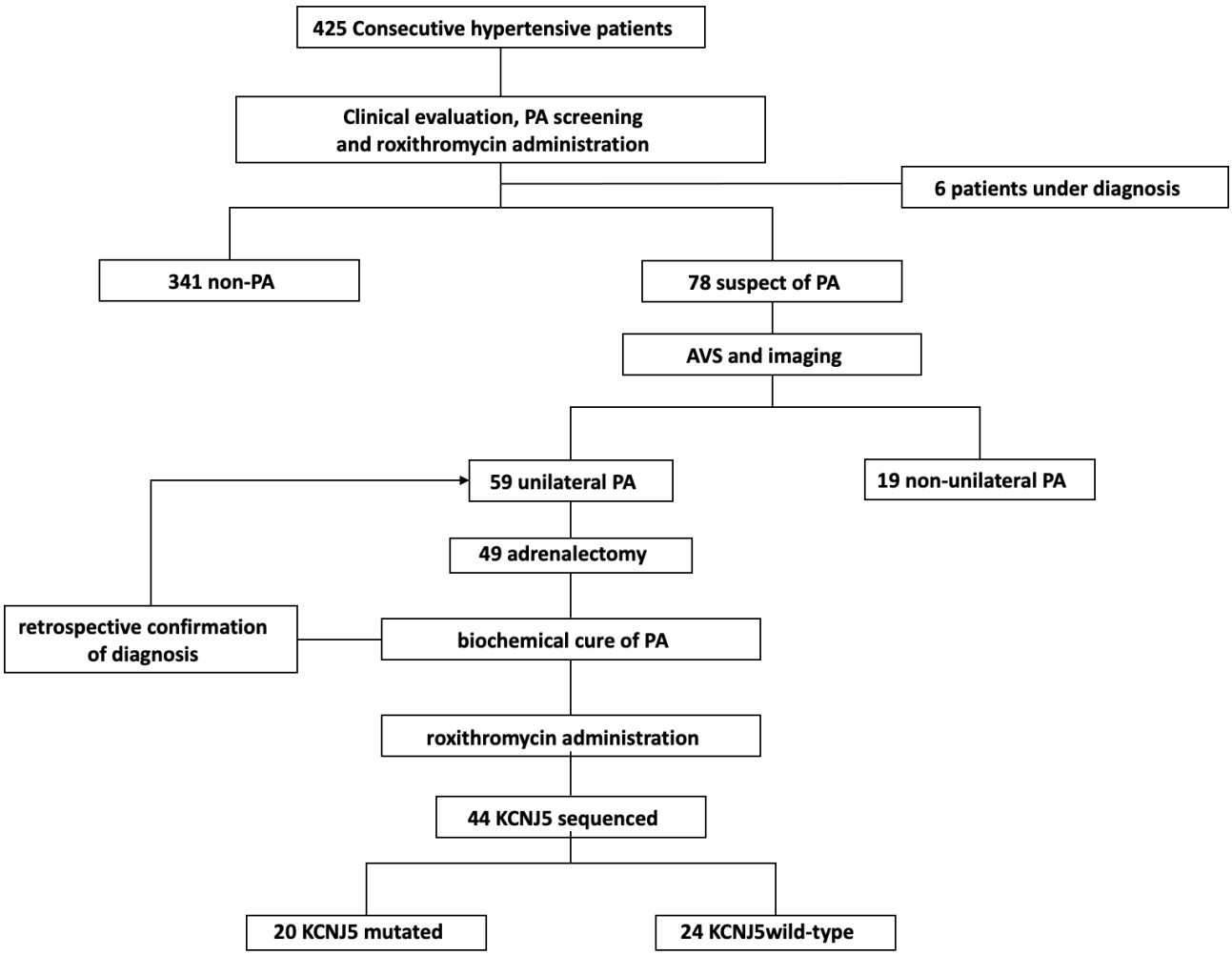

Figure 3 Flow chart of study design with numbers of enrolled patients that complete the diagnosis protocol.

General baseline characteristics of the patients divided in non-PA, unilateral PA and non-unilateral PA, are reported in Table 1. Mean age of unilateral PA was significantly higher than in non-PA and non-unilateral PA (p=0.001). Patients were equally distributed between man and women in all groups; no differences have been found in BMI. Patients presenting with unilateral-PA showed higher blood pressure, both systolic (p<0.001) and diastolic (p=0.080) .

	non-PA	**non-unilateral PA**	**unilateral PA**	**p value**
N.	341	19	59	
Age, years (SD)	48.2 (12.7)	55.2 (11.7)	53.4 (9.6)	**0.001**
BMI, kg/m^2 (SD)	26.4 (4.2)	27.3 (4.4)	27.3 (6.9)	0.451
Gender – male, %	59.8	84.2	66.1	0.079
SBP, mmHg	138 (15.5)	145 (18.8)	148 (17.4)	**< 0.001**
DBP, mmHg	87 (9.4)	87 (10.9)	91 (12.0)	0.080
Na^+, mmol/L (SD)	140.9 (1.8)	141.4 (2.4)	142.6 (2.3)	**< 0.001**
K^+, meq/L (SD)	4.0 (0.4)	3.5 (0.5)	3.6 (0.5)	**< 0.001**
ACE inhibitors, %	93.8	94.7	90.7	0.707
ARBs, %	67.7	73.7	74.1	0.610
CCBS, %	9.7	21.1	5.6	0.144
Diuretics, %	86.6	78.9	85.2	0.654
MRA, %	9.8	100	85.2	**<0.001**
PAC, ng/dL (SD)	9.9 (6.4)	19.3 (12.2)	29.8 (18.2)	**< 0.001**
DRC, mIU/L (SD)	18.3 (94.3)	13.0 (16.9)	4.1 (5.2)	0.502
PCC, nmol/L (SD)	255.9 (99.3)	314.2 (147.6)	253.7 (101.7)	0.051
ARR, ng/dL/mIU/L (SD)	1.7 (2.3)	4.0 (3.4)	11.2 (9.5)	**< 0.001**

Table 1 ANOVA comparison of general characteristics of population divided in non-PA, non-unilateral and unilateral forms of PA. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; Na^+, serum sodium; K^+, serum potassium; ACE inhibitors, angiotensin converting enzyme inhibitors; ARBs, angiotensin receptor blockers; CCBs, calcium channel blockers; MRA, mineralocorticoid receptor antagonists; PAC, plasma aldosterone concentration; DRC, direct renin concentration; PCC; plasma cortisol concentration; ARR, aldosterone renin ratio.

Patients presenting unilateral-PA showed higher serum sodium (p<0.001) and lower serum potassium (0<0.001). No differences were found concerning drug therapy except for MRA use that was more frequent in bilateral and unilateral PA group (p<0.001) (Table 1).

Patients presenting unilateral forms of PA showed a higher production of aldosterone (p<0.001) with consequent renin suppression. Plasma cortisol concentration was higher in non-unilateral forms of PA (p=0.051). ARR was higher in unilateral than in non-unilateral forms of PA (p<0.001) (Table 1).

After the first 200 cases, based on a preliminary analysis and discussion with the clinical pharmacologist it was decided to change the dose of roxithromycin from 150mg to 300mg to obtain an increase in plasma concentration (Figure 4). All data has been analyzed separately for 150mg and 300mg. As the results in different groups were the same between the two dose of roxithromycin, and in order to obtain more robust analysis, we decided to present results by combining 150mg and 300mg together.

Figure 4 number of enrolled patients that took 150mg, 300mg of roxithromycin. Patients in the middle took both doses during the diagnostic work-up.

Even if a small percentage of patients did not achieve the IC50 measured in vitro (0.53 – 1.29 mcg/mL), the dose switch leads to a significant increase in plasma roxithromycin concentration as reported in Figure 5. These patients that did not absorb the macrolide were excluded from following analysis.

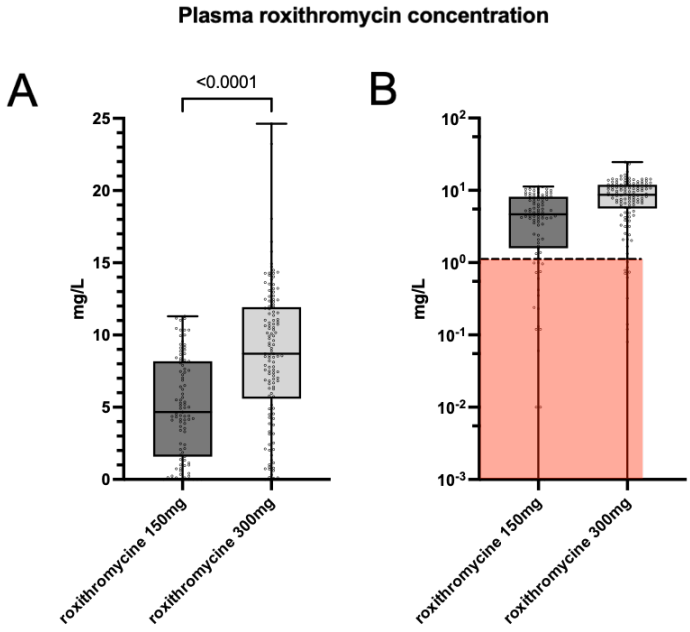

Figure 5 A) roxithromycin concentration measured in plasma. B) roxithromycin concentration in logarithmic scale, measured in plasma and showing the IC50 cut-off measured in vitro (0.53–1.29 mcg/mL).

In non-PA subjects the administration of roxithromycin decrease aldosterone secretion (mean change -0.72ng/dL; p<0.001) as reported in Figure 6. Moreover, a significant decrease in DRC (mean change 0.70 mIU/L; p<0.001) and PCC (mean change -47nmol/L; p<0.001) has been observed in these patients (Figure 6). Systolic blood pressure showed a significant decrease (mean change -2mmHg; p<0.001) while no effects has been observed on diastolic blood pressure.

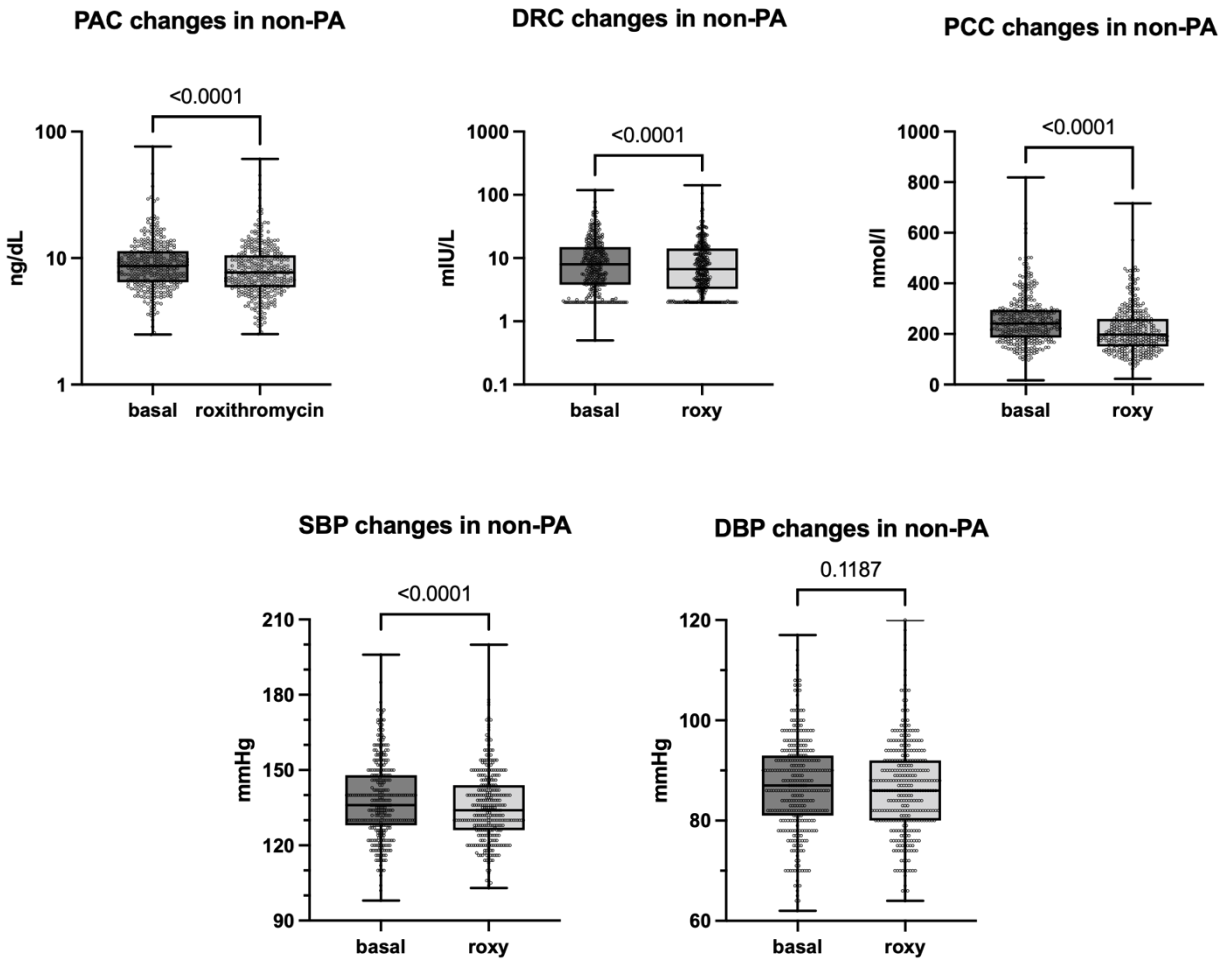

Figure 6 Changes in PAC (axis in logarithmic form), DRC, PCC, SBP and DBP after roxithromycin administration in patients with non-PA. PAC, plasma aldosterone concentration; DRC, direct renin concentration; PCC; plasma cortisol concentration; SBP, systolic blood pressure; DBP, diastolic blood pressure.

When comparing basal and post-roxithromycin in non-unilateral forms of PA, no effects has been observed in PAC or DRC while a significant decrease in PCC (mean change -66.5nmol/L; p=0.006) was observed. Systolic and diastolic blood pressure did not change in response to roxithromycin administration; if systolic blood pressure even showed a trend to decrease (Figure 7).

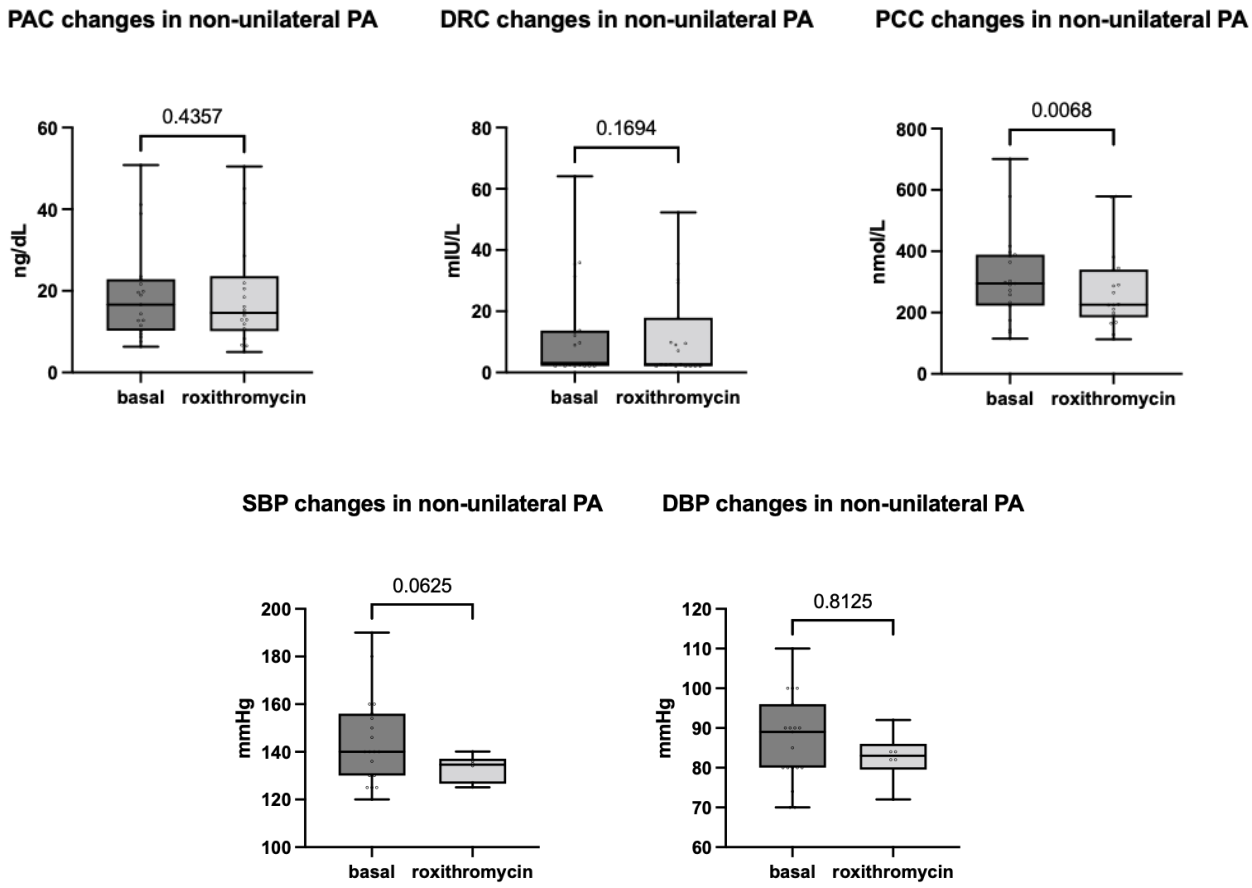

Figure 7 Changes in PAC, DRC, PCC, SBP and DBP after roxithromycin administration in patients with non-unilateral forms of PA. PAC, plasma aldosterone concentration; DRC, direct renin concentration; PCC; plasma cortisol concentration; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Genetic analysis identified 20 patients with a KCNJ5 mutant APA (Figure 8): 9 patients had a G151R KCNJ5 mutation, while 1 had a L168R KCNJ5 mutation. Interestingly, 1 patient carried both mutations in KCNJ5.

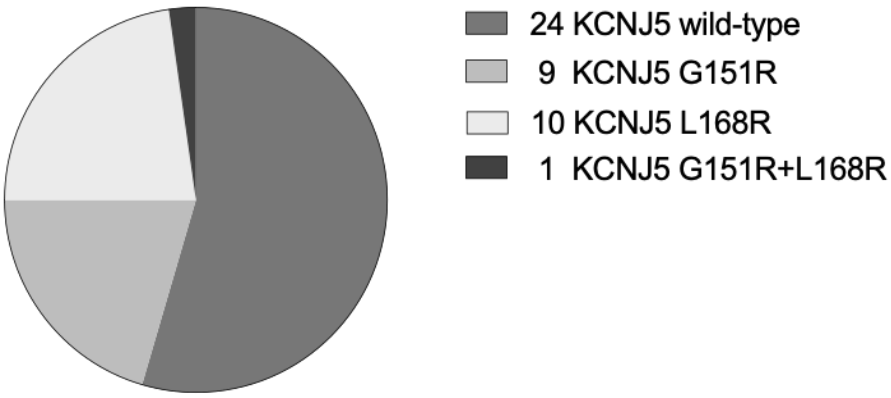

Figure 8 Results from KCNJ5 sequencing by NGS. Wild-type = 24 patients; KCNJ5 G151R = 9 patients; KCNJ5 L168R = 10 patients; KCNJ5 G151R+L168R = 1 patient.

	KCNJ5 wild-type	KCNJ5 mutated	p value
N.	24	20	
Age, years (SD)	58.0 (8.5)	49.1 (8.1)	**0.004**
BMI, kg/m^2 (SD)	27.6 (3.7)	25.4 (8.6)	0.358
Gender – male, %	19 (79.2)	12 (57.1)	0.102
SBP, mmHg	145 (14.5)	152 (21.5)	0.279
DBP, mmHg	88 (10.1)	93 (14.3)	0.323
Na^+, mmol/L (SD)	142 (1.3)	142 (1.7)	0.211
K^+, meq/L (SD)	3.7 (0.4)	3.3 (0.5)	0.379
ACE inhibitors, %	4.5	9.5	0.482
ARBs, %	31.8	28.6	0.540
CCBS, %	90.9	100	0.256
Diuretics, %	22.7	4.8	0.103
MRA, %	77.3	100	**0.027**
PAC, ng/dL (SD)	31.1 (23.5)	28.8 (15.2)	0.894
DRC, mIU/L (SD)	3.6 (2.8)	3.3 (2.2)	0.707
PCC, nmol/L (SD)	266.5 (124.4)	248.3 (71.1)	0.888
ARR, ng/dL/mIU/L (SD)	11.7 (11.7)	11.5 (8.3)	0.621

Table 2 general characteristics of KCNJ5 mutated and wild-type patients. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; Na^+, serum sodium; K^+, serum potassium; ACE inhibitors, angiotensin converting enzyme inhibitors; ARBs, angiotensin receptor blockers; CCBs, calcium channel blockers; MRA, mineralocorticoid receptor antagonists; PAC, plasma aldosterone concentration; DRC, direct renin concentration; PCC; plasma cortisol concentration; ARR, aldosterone renin ratio.

General characteristics of KCNJ5 mutated and wild-type patients were reported in Table 2. Patients with wild-type KCNJ5 were more frequent man and older if compared to mutated; no differences have been found in blood pressure, sodium and potassium levels. KCNJ5 mutated patients presented a more frequent use of mineralocorticoid receptor antagonists, while no differences have been found in use of other antihypertensive drug treatment.

KCNJ5 mutated patients showed a significant decrease in PAC (p=0.0305) after roxithromycin administration while in wild-type subjects no effect has been observed (Figure 9). The median difference between pre- and post-roxithromycin was -1.93 ng/dL (CI95%: -3.6 - +0.2). The amount of decrease is similar in almost all patients carrying KCNJ5 mutations with no differences related to G151R or L168R isoform (data not showed).

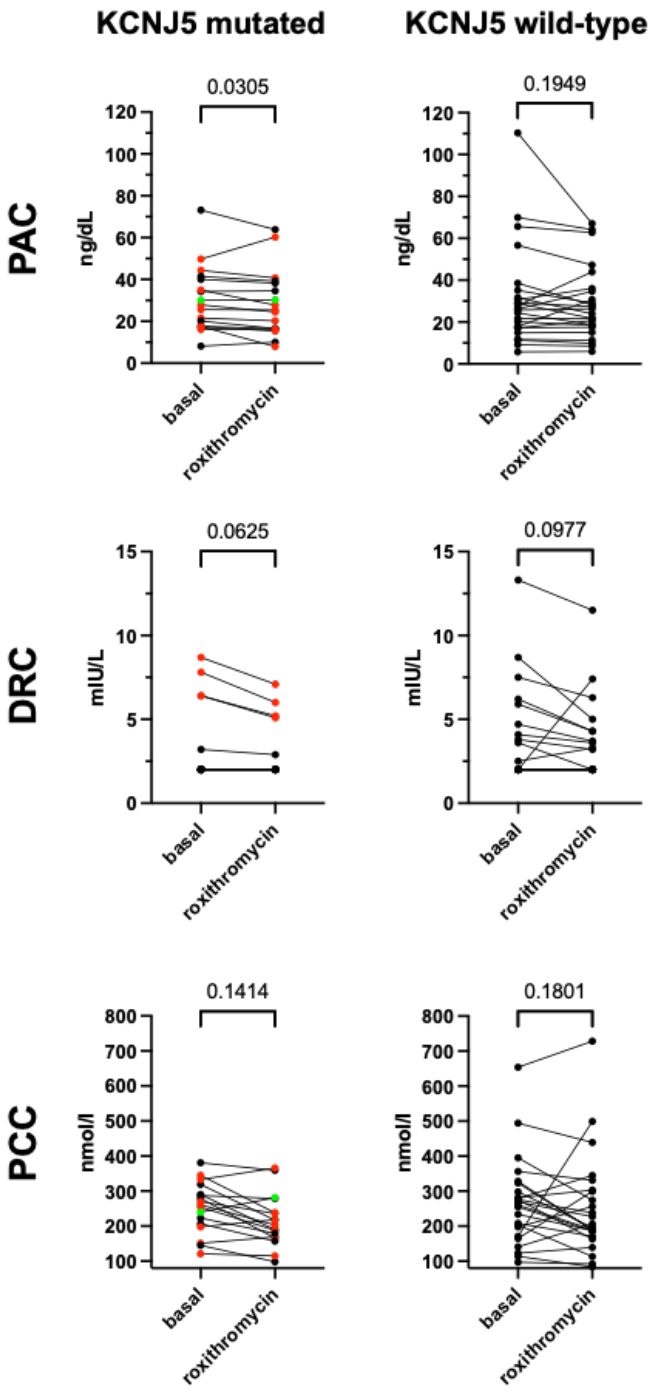

Figure 9 Effect of roxithromycin on PAC, DRC and PCC in KCNJ5 mutated and wild-type unilateral PA patients. Red points in KCNJ5 mutated patients represent G151R isoform, black points represent L168R, green point represent G151R+L168R mutation. Same values of different patients have been overlapped in graphs. PAC, plasma aldosterone concentration; DRC, direct renin concentration; PCC, plasma cortisol concentration.

DRC and PCC showed no differences in roxithromycin response in KCNJ5 mutated or wild-type patients (Figure 10). However, in patients carrying the mutation, the trend was homogeneous with

a general decrease in almost all patients; otherwise in wild-type KCNJ5 patients the behavior was heterogeneous, with some patients increasing while other decreasing. Both systolic and diastolic blood pressure did not significantly change in response to roxithromycin in patients with KCNJ5 mutations neither in wild-type (Figure 7). Interestingly, patient carrying G151R + L168R mutated KCNJ5, presented highest values of SBP and DBP

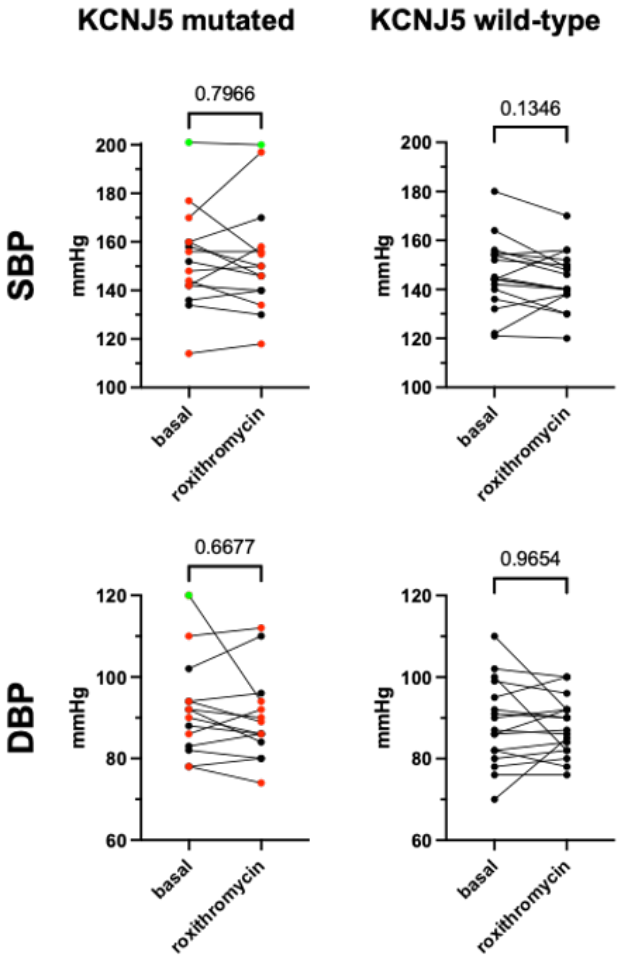

Figure 10 Effect of roxithromycin on PAC, DRC and PCC in KCNJ5 mutated and wild-type unilateral PA patients. Red points in KCNJ5 mutated patients represent G151R isoform, black points represent L168R, green point represent G151R+L168R mutation. Same values of different patients has been overlapped in graphs.

To verify the biochemical cure of unilateral PA patients and to test if the previous roxithromycin effect on PAC is related to the presence of mutated channel in APA, PAC levels of KCNJ5 mutated patients were analized. All subjects with unilateral form of PA demonstrated normalization of PAC levels after surgery. As hypotized, the inhibitory effect of roxithromycin on PAC after the adrenalectomy was not observed (Figure 11).

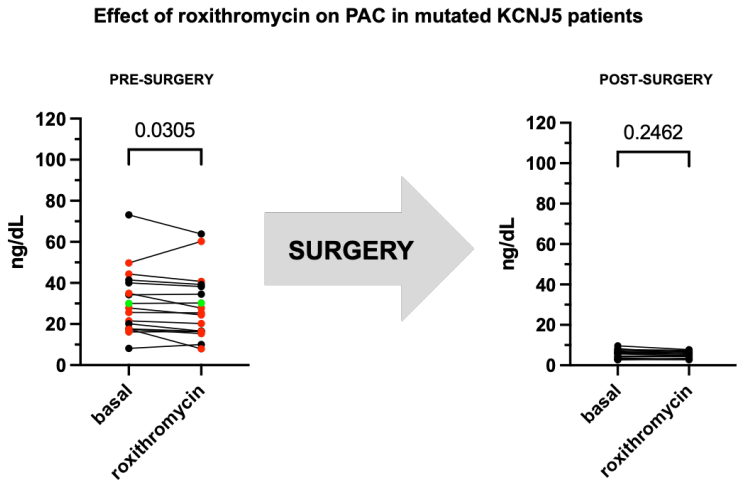

Figure 11 Effect of roxithromycin on PAC levels in mutated KCNJ5 patients, between pre- and post-surgery. Red points represent G151R isoform, black points represent L168R, green point represent G151R+L168R mutation.

DISCUSSION

Primary aldosteronism is the most frequent cause of endocrine hypertension affecting more than 11% of patients referred to specialized hypertension centers and over 20% of patients with resistant hypertension (Douma et al.). This is the first study that evaluate the effect of macrolide on aldosterone secretion and blood pressure changes in a large cohort oh hypertensive patients comprising unilateral PA, non-unilateral PA and non-PA. Patients has been prospectively enrolled in a Center of Excellence of the European Society of Hypertension for more than 4 years. In our cohort, PA accounts for 18% of cases, a prevalence consistent with those described in literature (Funder and Carey).

Patients with a biochemical suspect of PA underwent to AVS and the majority of the unilateral forms of PA showed lateralization. This data suggests, once more, that AVS is a crucial step to identify unilateral form from the non-unilateral. Prevalence of PA in unilateral and non-unilateral forms, and of non-PA reported in this study is highly representative of patients that refers to referral centers for hypertension. In these patients, we found a 45% of cases with KCNJ5 mutation. This prevalence is in line with the 43% described by Lenzini *et al.* in a meta-analysis involving data from different European centers (Lenzini, Rossitto, et al.).

The change of roxithromycin dose from 150mg to 300mg increase the macrolide plasma concentration allowing not only to raise the IC50 measured *in vitro*, but also the peak plasma concentration defined by pharmacological study (5 – 12ug/mL). The oral *via* involves different variables in absorption, plasma protein binding, distribution etc., consequently it is acceptable that a small part of cohort did not achieve the IC50.

The roxithromycin administration induce a decrease in aldosterone production in patients with KCNJ5 mutation but not in wild-type. This data confirm that macrolides are able to blunt aldosterone production by blocking the mutated Kir3.4 sodium channel without affecting wild-type forms. No differences of terms of inhibition power have been found between G151R and L168R isoforms (data not showed). The inhibitory effect of roxithromycin may be attenuated not only by the low dose of drug administered, but mostly by the single dose. These aspects may explain the fact that both systolic and diastolic blood pressure did not change in response to roxithromycin. Given that aldosterone acts by the renin angiotensin system that can be considered a long-term blood pressure regulation, the acute decrease of aldosterone was not enough to affect blood pressure. According to our hypothesis, in mutated KCNJ5 patients the inhibitory effect of

roxithromycin on PAC was strictly related to the presence of the tumor. In fact, after adrenalectomy the administration of roxithromycin did not induce any change in PAC.

This study present some limitations. First of all, roxithromycin was able to induce a decrease in PAC also in non-PA patients. This variation may be related to a stress-resolution effect. In fact, in those patients we observed a decrease also in PCC and in systolic but not diastolic blood pressure. As the pro-hypertensive effect of aldosterone is less marked in those patients, it may be possible that the stress-resolution induce a decrease in PAC with consequent decrease in blood pressure. To date there are no data concerning possible effects of macrolides on PAC in primary hypertensive patients. This finding, however, open new horizon in investigating possible pathways involved in macrolides-PAC or blood pressure regulation as suggested by Akatin *et al.* (Aktan et al.). Another limitation is related to the low dose of roxithromycin used and the single oral dose administered. Even if we measured the plasma roxithromycin concentration, the drug needs to be metabolized, absorbed, distributed, and once binded to the mutated channel it is necessary time to decrease the already-secreted aldosterone. All those variables may affect the efficacy of roxithromycin in blunting aldosterone production in KCNJ5 mutated unilateral PA forms. However, we have provided different control group of consecutive hypertensive patients, both PA and non-PA, to evaluate possible effect of roxithromycin not related to KCNJ5 mutation. Moreover, having a matched PAC, DRC and PCC group of unilateral PA KCNJ5 wild-type patients, we can assume the observed effect of roxithromycin is independent from possible confounders.

To conclude, roxithromycin administration induce a decrease in aldosterone production in patients with KCNJ5 mutation but not in wild-type, confirming its ability *in vivo* to blunt aldosterone production by blocking the mutated Kir3.4 sodium channel without affecting wild-type forms. As the decrease in PAC in the same patients did not occur after adrenalectomy, the roxithromycin effect is unequivocally attributable to the KCNJ5 mutated unilateral PA. However, as the decrease in PAC has not occurred strictly in KCNJ5 mutated unilateral PA, and due to the heterogeneity of the decrease, roxithromycin administration cannot be considered a tool for screening of KCNJ5 mutated APA yet. However, further studies involving KCNJ5 mutated unilateral PA with a higher dose and time administration of roxithromycin may clarify the role of this macrolide in aldosterone production and blood pressure regulation mechanisms.

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CHAPTER 2

CARDIOVASCULAR RISK PROFILE AND EARLY VASCULAR DAMAGE IN PATIENTS WITH COMMON VARIABLE IMMUNODEFICIENCY

CONTENTS

ABBREVIATIONS

ACS acute coronary syndrome

ASCVD atherosclerotic cardiovascular disease

AIHA autoimmune hemolytic anemia

AIX augmentation index

AP augmentation pressure

BCR B-cell receptor

BMI body mass index

BP blood pressure

CKD chronic kidney disease

CRP C-reactive protein

CV cardiovascular

CVID common variable immunodeficienc

DBP diastolic blood pressure

ddPCR droplet digital PCR

eGFR estimated glomerular filtration rate

ESID european society of immunodeficiency disease

GAPDH glyceraldehyde 3-phosphate dehydrogenase

GLILD granulomatous lymphocytic interstitial lung disease

HbA1c glycated hemoglobin

HDL high density lipoprotein

HR heart rate

ICON international consensus document

ICOS inducible co-stimulator

IgA immunoglobulin A

IgG immunoglobulin G

IgM immunoglobulin M

IGRT immunoglobulin replace therapy

IMT intima-media thickness

ITP autoimmune thrombocytopenia

IVIG intravenous immunoglobulin

LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LVH	left ventricular hypertrophy
MAP	mean arterial pressure
MR	mineralocorticoid receptor
NGS	next generation sequencing
OSE	oxidation-specific epitopes
OXLDL	oxidized low-density lipoprotein
PAD	peripheral artery disease
PAD	primary antibody deficiency
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PID	primary immunodeficiency disorders
PP	pulse pressure
PWA	pulse wave analysis
PWV	pulse wave velocity
RSV	respiratory syncytial virus
SBP	systolic blood pressure
SCIG	subcutaneous immunoglobulin
SD	standard deviation
SR	scavenger receptor
TIA	transitory ischemic event
VTE	venous thromboembolism
WHO	world health organization

ABSTRACT

Introduction: Although the immune system is involved in vascular disorders, the actual role of B cells in atherosclerotic cardiovascular disease (ASCVD) remains unclear. Inflammatory conditions like Rheumatoid Arthritis and Systemic Lupus Erythematosus present an accelerated atherosclerotic process, that is somehow limited by appropriate treatment, including B cell depleting therapies. Common Variable Immunodeficiency (CVID) is a rare primary immunodeficiency of adulthood and represents a pathological condition suitable for studying the role of B cells in ASCVD. The cardiovascular risk profile of patients affected by this rare disease is unexplored and it is unclear whether CVID patients are protected towards atherogenesis.

Aim: We investigated the prevalence of cardiovascular risk factors and the presence of subclinical ASCVD in CVID patients.

Methods: We collected anamnestic clinical and biochemical data related to CVID and to ASCVD in a single center cohort of CVID patients, grouped according to clinical and immunological phenotype. At follow-up visit vascular structural and functional investigation was performed by SphygmoCor(R) XCEL instrument while droplet digital PCR analysis was used to assess gene expression.

Results: 127 CVID patients were enrolled in the study. Patients with complicated phenotype presented significantly lower levels of cholesterol and blood glucose, despite a significantly higher use of corticosteroids ($p=0.014$) due to higher frequency of GLILD ($p<0.001$) and autoimmunity($p=0.007$), particularly ITP ($p<0.001$). Patients with Chapel phenotype 2 and 3 presented the lowest cholesterol and HbA1c levels. No significant difference was found in terms of metabolic syndrome, hypertension, diabetes, anti-hypertensive, anti diabetic and lipid lowering ongoing treatment, as well as acute cardiovascular events between complicated and uncomplicated phenotype. Lower IgA, IgM IgG levels at diagnosis, SmB cells % as well as higher T-LGL % (CD3+CD8+CD57+) and monthly dosage of IgRT in the complicated group were the main between groups significantly different immunologic parameters. In vivo measurement by SphygmoCor(R) XCEL instrument in a subgroup of 55 patients showed significant difference pulse pressure and augmentation pressure, with a tendence towards higher arterial thickness in patients with only infections. In the same subgroup, B lymphocytes isolated from pheripheral blood of patients with complicated phenotype presented a borderline-significantly higher expression of LDL receptor gene at droplet digital PCR analysis ($p=0.053$).

Conclusion: Our data suggest that clinical phenotypes of CVID may be associated with different cardiovascular risk profiles, possibly based on the different underlying immunological alterations. Preliminary data suggests that B cell defects in CVID patients might influence the development of cardiovascular disease leading to different cardiovascular risk profile. The role of IgRT also needs to be explored. Follow-up studies will unravel the significance of our subclinical findings in terms of development of overt disease, with possible implications in terms of new therapeutic strategies for ASCVD.

BACKGROUND

PRIMARY IMMUNODEFICIENCY

Primary Immunodeficiency Disorders (PIDs) are a group of diseases comprising more than 400 immunity disorders distinguished by different degrees of severity. They are characterized by increased susceptibility to infections, autoimmunity phenomena, inflammatory diseases, allergy and cancer. PIDs are caused by monogenic germline mutations or a combination of genetic and/or epigenetic alterations that can result in loss of expression, functional alteration, loss of function or gain of function of encoded proteins. These proteins play a crucial role in the development, maintenance, and function of the immune. Primary immunodeficiencies are rare diseases. In recent years, the application of the latest gene sequencing techniques has identified a total of 430 recognized gene defects associated with primary immunodeficiency. Nevertheless, the prevalence in the general population remains difficult to estimate, both because these diseases are still underdiagnosed and because of the different approach to the use of registries (Bucciol and Meyts). Among these, the ESID (European Society of Immunodeficiency Disease) reports that the overall prevalence of these diseases is estimated to be between 1/1000 and 1/5000 (B. Gathmann et al.).

Primary antibody immunodeficiencies include a wide range of disorders characterized by the inefficient production of a clinically effective antibody response. The primary deficiency quantitatively or qualitatively compromises the integrity of the B-cell compartment and can affect any stage of the B-lymphocyte maturation process. B-lymphocyte maturation begins from lymphoid precursors present in the bone marrow, and through a series of rearrangement processes at the level of the immunoglobulin genes, each lymphocyte produces a B-cell receptor (BCR) capable of recognizing a large variety of antigens. At the level of secondary lymphoid organs, the maturation process ends with the presentation of the antigen to the naïve B-lymphocyte, which is activated by undergoing the process of isotypic recombination and somatic hypermutation thanks to which immunoglobulins of different classes with high affinity are produced. At the end of this process, differentiation into plasma cells and memory B cells occurs (Figure 1) (Devonshire and Makhija).

The phenotype characterizing Primary antibody immunodeficiencies is highly variable, as it ranges from disorders characterized by a marked reduction of B lymphocytes and all classes of immunoglobulins to deficits in antibody response limited to specific antigens. The most frequent clinical manifestation is recurrent infectious episodes, typically in the airways and in the gastrointestinal tract. Also relevant are the phenomena associated with immune system dysregulation, which determine an increased incidence of autoimmune manifestations, allergy, lymphoproliferative disease and cancer (Cinetto et al.).

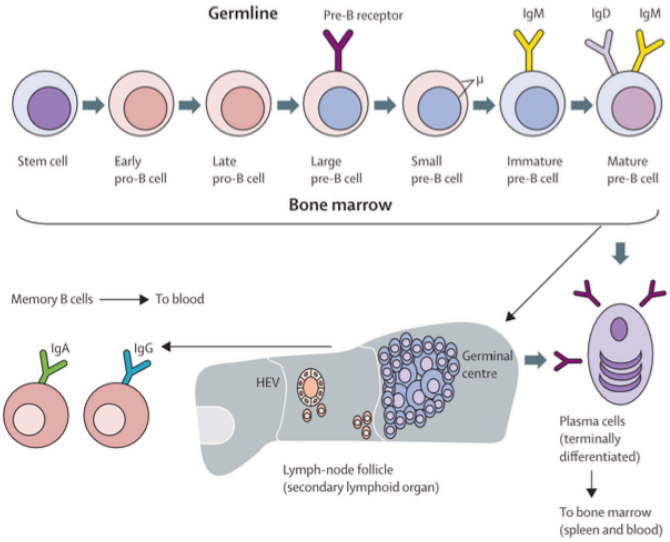


Figure 1 B lymphocyte maturation stages. By M. Park, Expert Review of Clinical Immunology 2011

COMMON VARIABLE IMMUNODEFICIENCY

The definition of common variable immunodeficiency was proposed in 1971 by the World Health Organization (WHO) to indicate a set of syndromes characterized by hypogammaglobulinemia, with the aim of distinguishing them from primary antibody immunodeficiencies with autosomal recessive presentation and a more specific clinical phenotype. Today, according to the International Consensus Document (ICON), common variable immunodeficiency is defined by the following characteristics (Bonilla et al.):

- marked decrease of IgG (at least 2 SD below the mean for age) and a marked decrease in at least one of the isotypes IgM or IgA;
- age greater than 2 years;
- absence of isohemagglutinins and/or reduced or absent vaccine response;
- exclusion of other causes of hypogammaglobulinemia

CVID is the most frequent symptomatic primary antibody immunodeficiency in adults. Selective IgA deficiency has higher prevalence, but most patients are asymptomatic. It equally affects both genders and the prevalence is estimated to be between 1:25000 and 1:50000 (Bonilla et al.).

Symptoms generally onset between the ages of 20 and 40 years, although it can begin at any age. A diagnostic delay of about 6 to 7 years on average has been observed but can exceed 15 years in up to 20% of patients (Cunningham-Rundles and Bodian) (Benjamin Gathmann et al.). Most patients with onset before the age of 10 years are male and, in these cases, other X-chromosome-related causes of hypogammaglobulinemia must be excluded by genetic analysis (Conley et al.). Most cases of CVID are sporadic, while only 5-25% have a familial pattern. In these cases, there is generally a family history of selective IgA deficiency (Bonilla et al.).

PATHOPHYSIOLOGY

The heterogeneity of clinical manifestations of CVID can be explained by the presence of numerous gene defects/polymorphisms that contribute to the development of the clinical phenotype. With the rapid development of Next Generation Sequencing (NGS) it has been possible to identify numerous genes leading to an increasingly detailed understanding of the pathogenesis of this condition (Tangye et al.).

In about 2% of cases, a monogenic defect with autosomal recessive transmission is identified. The search for such mutations is not necessary for diagnosis and therefore is not part of the ESID diagnostic criteria (ESID Registry Working Party); however, it is suggested if there is a positive family history of primary immunodeficiency. To date, the genes that have been associated with CVID are: CD19, CD20, CD21, CD27, CD81, CTLA4, ICOS, IL21, IL21R, LRBA, NFKB2, PIK3CD, RAC2, TWEAK. In addition to specific gene defects, numerous polymorphisms associated with increased susceptibility to develop CVID have been identified. Among these, most involve the HLA haplotypes DQ2, DR7, DR3, B8 and/or B44. Other polymorphisms among the most studied are those of the TACI/TNFRSF13B (transmembrane activator and calcium-modulating cyclophilin ligand interactor) gene, which are found in 5-8% of patients with CVID, although they are present in about 1% of healthy individuals. Most individuals with polymorphisms in the TACI gene manifest the disease, while those with the mutation in the single allele present an increased risk of developing CVID and autoimmune manifestations (Amatore et al.). The development and maturation of B lymphocytes are dependent on the BCR (B- cell receptor)-activated signaling pathway, and for this reason mutations in genes involved in signal transduction of this pathway may cause an inability to produce an effective humoral response (van Zelm et al.). The TACI protein interacts with BAFF and/or APRIL. These interactions are crucial for the B-cell function as they enable survival, immunoglobulin production and isotype switching. Alterations at the level of these genes result in a reduction in the B-cell subpopulation called switched memory (smB) that is associated with an increased risk of developing autoimmunity and splenomegaly (Knight et al.) (Varzaneh et al.).

The inducible costimulator on activated T-cells (ICOS) gene encodes for a costimulatory molecule belonging to the CD28 family that is expressed by activated T lymphocytes. This interacts with ICOS-L on B lymphocytes, inducing their activation and proliferation. A mutation in ICOS results in an arrest of activation, proliferation, and maturation of memory B-cells, resulting in a reduced level of peripheral B lymphocytes and nearly absent memory B lymphocytes (Amatore et al.). In most patients with ICOS deficiency, clinical signs of disease occur in adulthood, whereas a normal

lymphocyte count is present in pediatric age, which tends to progressively decrease in adulthood. These patients have an increased susceptibility to develop autoimmune manifestations, lymphoproliferation and splenomegaly (Deane et al.).

PATHOGENESIS

To date, the pathogenesis of CVID is still partly unknown. The deficit of humoral immunity characteristic of this disease is to be found in the late stages of B lymphocyte maturation and differentiation of B lymphocytes into effector phenotypes. The defect may thus be caused by an intrinsic B lymphocyte deficiency or by an alteration in T lymphocyte function affecting B lymphocyte maturation. Indeed, in many patients, there are alterations in CD4+ T lymphocytes that result in an alteration in their function. Recently, other possible alterations involving maturation at the level of the thymus, development of dendritic cells and macrophages and the reduction of NK cells have also been demonstrated. Given the complexity and the number of mechanisms involved in the pathogenesis, however, it is difficult to distinguish primary from secondary cellular alterations (Bonilla et al.).

EUROclass CLASSIFICATION

Classification of CVID patients into subgroups on the basis of B lymphocyte subpopulations identifiable by cytofluorimetry techniques began in 2002 through the work of Warnatz et al. who produced the Frieburg classification (Warnatz et al.). A further classification (Paris classification) was proposed in 2003 (Piqueras et al.). A consensus was finally reached in 2008 with the EUROclass (Wehr et al.), which combines the previous classification schemes. Patients can then be divided into two categories based on the presence (B+) or absence (B-) of B lymphocytes. Presence is defined by a percentage greater than or equal to 1% of the total number of lymphocytes. B- patients account for about 10% and are associated with a worse prognosis than B+ patients. The trial focuses on the analysis of the following B lymphocyte subpopulations excluding the group of B- patients:

- Naïve B lymphocytes - Naïve B lymphocyte counts are not associated with any clinical manifestations. For this reason, it is not included in the classification system.
- Switched-memory B lymphocytes (CD27+IgM-IgD-) (smB) - 80% of B+ patients have smB deficiency, defined by the presence of less than 2% of smB of total lymphocytes. This finding indicates the

presence of an alteration at the level of the germinal center. SmB- patients present on average lower levels of IgA and IgG than other patients. They also present with an increased incidence of splenomegaly and granulomatous disease.

- Activated B lymphocytes ($CD21^{low}CD38^{low}$): it is a population derived from memory B lymphocytes that have undergone repeated antigenic stimulation. In fact, these lymphocytes exhibit class switching, somatic hypermutation, activation markers, and poor proliferation. They are defined by the presence of more than 10% $CD21^{low}$ B lymphocytes (Visentini et al.). They are increased in patients with chronic infectious states and in some autoimmune diseases (Azizi et al.). An increased incidence of respiratory infections has also been demonstrated in this subgroup of patients (Moratto et al.).
- Transitional B lymphocytes (CD38++ IgM^{high}): precursors of mature B lymphocytes that have left the bone marrow. In a subgroup of smB- patients, a high number of transitional B lymphocytes (Trhi) defined by the presence of more than 9% transitional of total B lymphocytes was found. The latter phenotype is associated with the presence of lymphadenopathy in more than 50% of cases (Wehr et al.).

CLINICAL FEATURES

Increased susceptibility to infections is one of the main features of CVID. This characteristic is found in almost all patients. In an Italian multicenter study 86.7% of patients had a history of recurrent infections (Quinti, Soresina, et al.). The most common infections involve the upper and lower respiratory tract and are represented by bronchitis in 69% of cases, sinusitis (60%), pneumonia (58%), and over time patients may develop bronchiectasis (38%) (Quinti, Soresina, et al.). Infections can be acute, chronic or recurrent and are most often caused by capsulated bacteria such as *Streptococcus pneumoniae* and *Haemophilus influenzae* Type B. Other frequently isolated bacteria include *Neisseria meningitidis*, *Moraxella* spp., *Staphylococcus* spp., *Streptococcus* spp., *Pseudomonas aeruginosa*, and *Mycoplasma* spp. As for viral infections, the viruses most often encountered are Rhinovirus, Adenovirus, Coronavirus, Influenza virus A and B, Enterovirus, RSV (Respiratory Syncytial Virus), and Metapneumovirus (Benjamin Gathmann et al.) (Park et al.). Rhinovirus infection in patients with CVID is persistent and affects the lower respiratory tract. Chronic infections are associated with a reduction in host immune defenses, which can result in the establishment of inflammation that can lead to the development of bronchiectasis (Kainulainen et al.).

Patients with CVID also have an increased susceptibility to gastrointestinal infections that tend to become chronic and are difficult to eradicate despite instituting adequate antibiotic therapy. The most frequently isolated pathogens are *Giardia lamblia*, *Campylobacter jejuni*, *Salmonella* spp. and more rarely CMV. Infections caused by Norovirus and *Campylobacter jejuni* can cause episodes of acute diarrhea, while the forms caused by *Giardia* and CMV can manifest with chronic pictures, malabsorption, weight loss, or growth retardation. Urinary tract infections caused by *Ureaplasma urealyticum* or *Mycoplasma* (Chapel et al.).

Pulmonary complications are the leading cause of morbidity in patients with CVID (Park et al.). In fact, they are associated with recurrent hospitalizations and significant mortality. Lung disease can result in obstructive and/or restrictive impairment and increased susceptibility to infection (Benjamin Gathmann et al.). The most frequently diagnosed autoimmune manifestations are autoimmune thrombocytopenia (ITP) affecting about 7% of patients, autoimmune hemolytic anemia (AIHA) affecting about 4% of patients or a combination of the two (Evans syndrome), and finally autoimmune neutropenia (Quinti, Soresina, et al.). Cytopenias may be the onset manifestation of CVID in about 10% of cases (Oksenhendler et al.). A lower incidence of infection associated with a higher frequency of splenomegaly, enteropathy, and granulomatous disease has been described in these patients (Park et al.).

Manifestations involving the gastrointestinal tract can be infectious or noninfectious in nature. It is estimated that up to 50% of patients may suffer from chronic diarrhea with malabsorption or intermittent diarrhea during the course of the disease, however, the symptoms are not diriment, so the classification is complex (Uzzan et al.). Noninfectious enteropathy affects 9-15% of patients with CVID (Saifi and Wysocki) and is defined by specific histopathologic findings: villous atrophy and alteration of mucosal crypts, predominantly CD8+ lymphocytic infiltration, lymphoid hyperplasia organized in aggregates in the wall, and plasma cell deficiency. These features turn out to be quite similar to celiac disease from which, however, enteropathy in the course of CVID differs in the absence of the antibodies typical of celiac disease (anti-transglutaminase, anti-endomysium, and anti-gliadin), the absence of the HLA-DQ2 and HLA-DQ8 haplotypes, and the lack of response to the gluten-free diet (Saifi and Wysocki) (Bonilla et al.) (Cunningham-Rundles and Bodian). Enteropathy is often found to be associated with autoimmune pictures; in fact, patients have other autoimmune disorders in 46% of cases and increased T lymphocytes in peripheral blood in 52% (Malamut, Verkarre, et al.). Hepatic involvement is strongly associated with enteropathy in the course of CVID

(Song et al.) (Malamut, Verkarre, et al.). The most frequently seen pathological pictures are reactive nodular hyperplasia, granulomas, and portal vessel abnormalities (Malamut, Ziol, et al.).

Granulomatous disease is defined by the demonstration by biopsy of at least one granuloma in one or more organs. The most affected organs are the lung, lymph nodes, spleen, and liver. Other organs that may be affected are the skin, bone marrow, kidneys, and encephalon. It affects about 10-20% of patients with IDCV. The histopathologic finding consists of noncaseous granulomas similar to those found in sarcoidosis, which is distinguished by the size and location of the granulomas, which in the case of sarcoidosis are typically perilymphatic and apical in distribution, the presence of hypergammaglobulinemia, and the less frequent association with splenomegaly (Park et al.) (Park et al.) (Verbsky and Routes). The presence of granulomatous disease is associated in 54% of cases with other autoimmune manifestations, especially cytopenia. In patients with granulomatous disease, a reduction in B lymphocyte switched memory has also been observed, which is associated with an altered cytokine environment, an increase in CD8+ T lymphocytes, and a reduction in CD4+ T lymphocytes, which would favor the development of granulomatous-type reactions (Ardeniz and Cunningham-Rundles). Patients with CVID have a 12- to 18-fold higher risk of developing malignancies than the general population (Tak Manesh et al.). The most frequent cancers are non-Hodgkin's lymphomas and gastric carcinoma. Malignancies are a major cause of death in patients with IDCV (Quinti, Agostini, et al.). Lymphomas in patients with CVID onset between the fourth and seventh decades and are more frequent in the female sex (Martín-Mateos and Piquer Gibert). Lymphoma is the most influential complication in terms of survival in patients with CVID (Chapel et al.). Gastric carcinoma is the second most frequent type of cancer in patients with CVID. Gastric carcinoma has distinctive features that differentiate it from other gastric neoplasms. First, it is diagnosed in patients on average younger than in the general population; second, the histologic diagnosis is of intestinal adenocarcinoma of a moderately/poorly differentiated tubular type that contains a high intratumoral lymphocyte count. Finally, it is described how this tumor arises on a particular type of atrophic gastritis found in patients with immunodeficiency and autoimmune disorders (De Petris et al.).

CLINICAL PHENOTYPES ACCORDING TO CHAPEL CLASSIFICATION

Given the heterogeneity and complexity of the innumerable clinical manifestations in patients with IDCV, based on data obtained from a multicenter study, proposed a classification of patients into 4

subgroups according to noninfectious complications. Among excluded complications there were bronchiectasis which is related to pulmonary infections; splenomegaly which is an extremely nonspecific manifestation and sideropenic anemia which has a multifactorial pathogenesis. The clinical phenotypes were identified as follows (Chapel et al.):

I. No disease-related complications or manifestations limited to recurrent infections (infection only);
II. Autoimmunity (organ-specific manifestations and autoimmune cytopenias);
III. Polyclonal lymphocytic infiltration (otherwise unexplained granulomas, hepatomegaly, persistent lymphadenopathy, and lymphocytic interstitial pneumonia);
IV. Enteropathy not otherwise explained (proven by biopsy and insensitive to gluten)

83% of patients have a single clinical phenotype and this characteristic tends to be stable over time. By dividing patients into two groups (one without complications and one with complications), increased mortality was demonstrated in patients with complications. More specifically, each phenotype correlated with a specific survival rate. Phenotypes III and IV are associated with higher mortality; phenotype II is associated with a lower but still significant increase in mortality (Chapel et al.). These data were confirmed by a subsequent longitudinal study showing that the survival of patients with CVID without extra-infectious complications is 95% compared with the survival of patients with complications, which is around 42% (Resnick et al.). Phenotype III has also been shown to correlate with a 5-fold increased risk of lymphoid neoplasia and is associated with higher serum IgM levels (Chapel et al.). Considering the most recent reevaluations, the new phenotypes divide the study populations into patients without disease-related complications (62%-78%), with autoimmune cytopenias (6- 15%), with lymphoproliferation (6 - 15%), and with enteropathy (1-4%) (Chapel et al.).

IMMUNOGLOBULIN REPLACEMENT THERAPY

Immunoglobulin administration is the therapy of choice for the treatment of primary immunodeficiencies characterized by major humoral deficits because it reduces the incidence of infection and morbidity, prolongs survival, and more generally improves quality of life. Initial intramuscular formulations were found to be poorly tolerated and were therefore replaced by intravenous (IVIG) and subcutaneous (SCIG) formulations. To date, there are about 20 immunoglobulin formulations available that exhibit similar efficacy but differ in chemical and

physical properties and IgA content. These different characteristics influence the possible adverse effects and tolerability of therapy (Quinti, Soresina, et al.) (Paquin-Proulx and Sandberg) (Schwab and Nimmerjahn). The immunoglobulin titer required to obtain adequate IgG levels varies from patient to patient. Values above 5g/L have been shown to be sufficient to prevent severe pulmonary infections in patients with IDCV, but higher titers are associated with a further reduction in the frequency of pneumonia in patients on replacement therapy (Sriaroon and Ballow) (Orange et al.). ESID guidelines recommend a starting dose of 0.4-0.5 g/kg/month for IVIG and 0.4-0.6 g/kg/month for SCIG. Higher dosages are indicated in cases of poor infection control, bronchiectasis, some cases of enteropathy, and splenomegaly, in cases of weight gain and in pregnancy (ESID Registry Working Party) (Bonilla et al.). Intravenous and subcutaneous formulations show no difference in effectiveness in preventing infection. The route of administration is decided based on clinical data such as the amount of immunoglobulin to be administered, poor tolerability to IVIG, presence of venous access, and patient compliance (Sriaroon and Ballow). IVIGs, unlike SCIGs allow large formulated volumes of immunoglobulins to be administered. Therefore, they are administered every 3 to 4 weeks. However, the intravenous route has disadvantages; in fact, about one-third of patients may experience systemic side effects during or after the infusion (e.g., headache, malaise, nausea and vomiting, myalgias, hypertension or hypotension, abdominal pain) treatable with premedication of corticosteroids and antihistamines. In addition, due to the pharmacokinetics of the drug, a lowering of protective immunoglobulin levels may occur in some patients in the days leading up to the next infusion resulting in an increased risk of infection (Orange et al.). Compared with IVIG, subcutaneous therapy has several advantages; in fact, as it does not require venous access, it is more convenient to administer for the patient, who can self-infuse the drug via specific infusion pumps. Systemic side effects are also reduced, and serum immunoglobulin levels exhibit less fluctuation than intravenous therapy. However, SCIGs have lower bioavailability and allow smaller volumes of Ig to be administered, which is why more injection sites are often required (Orange et al.) (Shrestha et al.). To date, several methods of administration of SCIGs are available that are essentially identical in terms of efficacy. They are generally administered weekly or biweekly through an infusion pump in a single dose, or they can be infused by rapid administration of small volumes (rapid push), which takes less time and does not require infusion pumps. Another facilitated delivery system involves association with recombinant hyaluronidase, which degrades hyaluronic acid present in the extracellular matrix, facilitating immunoglobulin absorption. This

allows a larger volume of solution to be infused requiring only one infusion site every 3-4 weeks as in the case of IVIGs (Bonilla) (Bienvenu et al.).

IMMUNE SYSTEM AND ATHEROSCLEROSIS

Atherosclerosis is the pathophysiological mechanism most frequently found to be associated with cardiovascular disease. Atherosclerosis is defined as a chronic degenerative disease of large- and medium-caliber arteries with multifactorial etiology that is characterized by the sclerosis of a previous atheroma, a term for the accumulation of lipids in the subendothelial portion of the intima of arteries. During the process of atherosclerosis, the first lesions formed are so-called lipid streaks consisting of subendothelial deposition of lipids, foam cells (foam cells), and T lymphocytes (Hansson and Hermansson). Over time, the lesion evolves with the formation of apoptotic cells and cholesterol crystals that go on to form the necrotic core of the plaque. This structure is covered by a fibrous cap and has on its sides so-called "shoulders" infiltrated by activated T lymphocytes, B lymphocytes, macrophages and mast cells that produce pro-inflammatory mediators and enzymes. Over time, the plaque increases in volume progressively occluding the vessel lumen and eventually ulcerating, eventually leading to thrombus formation with subsequent ischemic manifestations at the tissue level. Hypercholesterolemia is one of the main factors determining the initiation of the atherogenic process, as it is precisely the accumulation of low-density lipoprotein (LDL) at the intimal level that results in the activation of endothelial cells and the subsequent recruitment of immune cells. LDL, which contains mainly esterified cholesterol and triglycerides, accumulates at the intimal level where it undergoes oxidative modifications caused by myeloperoxidases and lipoxygenases or by reactive oxygen species generated in the context of inflammatory processes. Peroxidation of fatty acid residues produces a series of reactive molecules that induce activation of endothelial and macrophage cells, which thus produce adhesion molecules and chemokines. The oxidized LDL (oxLDL) that are produced during this phase comprise a series of molecules that go on to form a set of new epitopes called oxidation-specific epitopes (OSEs) toward which the immune response, mediated in particular by T and B lymphocytes, is activated (Hansson and Hermansson). B lymphocytes can remove lipid antigens via a B cell receptor (BCR)-dependent pathway or via the LDL receptor (LDLR). LDL removal by LDLR may play a relevant role not only in B lymphocyte metabolism, but also in the presentation of antigens derived from modified LDL that could participate in the inflammatory process observed in atherosclerosis. In addition, defective

expression or internalization of LDLR results in increased plasma LDL, predisposing it to oxidation and thus contributing to the pathophysiology of atherosclerosis. Other receptors involved in lipid recognition by B lymphocytes are the receptor for the Fc portion of immunoglobulins (FcR), which recognizes Ig directed against neoepitopes generated by lipid peroxidation, and scavenger receptors (SR), which belong to the pattern recognition receptor (PRR) family and recognize a variety of molecules, including oxLDL (Echeverri Tirado and Yassin). To further investigate the process of atherosclerosis from a molecular and cellular perspective, several studies have been carried out that have highlighted the role of lymphocytes in the process of atherogenesis. In mouse models of atherosclerosis deficient for either the apolipoprotein E gene ($ApoE^{-/-}$) or the LDL receptor gene ($LDLR^{-/-}$) crossed with mice with combined T and B immunodeficiency, a significant reduction in atherosclerotic lesion size was demonstrated, while CD4+ T lymphocyte transfer aggravated atherosclerosis (Reardon et al.) (Zhou et al.). Other studies have investigated the role of B lymphocytes more specifically, showing that in splenectomized $ApoE^{-/-}$ mice, atherosclerosis is aggravated, whereas reinfusion of B lymphocytes from an $ApoE^{+/+}$ donor restores the atheroprotective action of B cells (Caligiuri et al.). However, again in a mouse model, depletion of B lymphocytes by administration of anti-CD20 monoclonal antibodies has also been shown to result in a significant reduction in the atherosclerosis process (Ait-Oufella et al.). Macrophages, T cells activation and cytokines release are known to induce the progression and complication of atherosclerotic plaques (Fatkhullina et al.). More recently, even B cells and antibody mediated-immunity have been implicated in atherogenesis and the risk of vascular events (Ucar et al.) (Lorenzo et al.). However, most of the available data come from murine models. B cells are typically found in lymphoid follicles containing T cells, DCs, and macrophages in the adventitia of the atherosclerotic aortas, suggesting the importance of a local immune response. Increased numbers of B cells have been shown also in perivascular adipose tissue of human coronary plaques, apparently correlating with the extent of the obstruction. Interestingly, B cells in human perivascular adipose tissues also include CD20+CD27+CD43+ B cells, previously proposed as human equivalent of murine B1 cells. However, analyses of infiltrating B cells of human carotid plaques demonstrated predominance of B2-like CD20þ activated plasmablasts. Notably, resident B cells exhibited frequently IgA or IgG isotypes, inverted k/λ light chain ratios and limited sets of hypermutated V_H regions, indicative of oligoclonal antigen-driven B-cell responses. In terms of antibodies, high baseline IgM levels have been reported to be associated with freedom of cardiovascular events, and an atheroprotective effect of anti-oxLDL IgM antibodies has been suggested. Pro-atherogenic

effects have been suggested instead for IgG and IgE, while no data are available on the possible role of IgA (Porsch et al.). Experimental depletion of B cells in mice has been reported to attenuate plaque development and to modulate T cell responses (Pattarabanjird et al.). Accelerated atherosclerosis has been reported in B cell-mediated systemic autoimmune diseases as SLE, in presence of a well-known burden of autoantibody production. B cell targeted therapies, as anti-CD20 or anti-BAFF, in this setting, have been shown to blunt atherosclerotic disease progression (Pattarabanjird et al.) (Kerekes et al.). Further experimental evidence in mice underlines the proatherogenic effect of B cell antigen presenting function and costimulatory molecules activation (Tay et al.). However, conclusive data about the impact of the before mentioned B cell functions on the risk of cardiovascular disease (CVD), as well as the influence on physiological endothelial homeostasis of B cell activation following inflammatory cascades in humans, is still missing.

At present, it is unclear whether subjects with CVID will undergo accelerated vascular damage due to an increased inflammatory burden or are instead protected towards atherosclerosis progression due to the peculiarity of B cell impairment. Available data suggest that patients with secondary antibody deficiency and inflammatory diseases like rheumatoid arthritis might have an increased risk of atherosclerotic cardiovascular disease. However, these complications are not included between the main causes of morbidity and mortality in patients affected by CVID. Lower circulating HDL cholesterol levels have been reported in a cohort of CVID patients, suggesting a correlation between lower HDL and a higher degree of inflammation but without any specific investigation on the related cardiovascular risk (Macpherson M.E. et al., 2019). On the other hand, a potential beneficial role of intravenous immunoglobulin on endothelial function has been recently hypothesized (Napoli et al.).

VASCULAR STIFFNESS

Arterial stiffness is the main cause of the progressive increase in SBP and PP and concomitant reduction in DBP after the age of 40 years. It predominantly afflicts the aorta and large elastic arteries, involving small muscular arteries to a lesser extent. With aging, degeneration of elastic fibers is associated with production of collagen and fundamental substance, often accompanied by calcium deposition and elastin fragmentation. These structural changes result in a progressive reduction in arterial distensibility; the sphygmic wave is faster while the reflex wave merges with

the systolic component of the incident wave causing an increase in SBP and PP and a reduction in DBP. (Laurent et al.) (O'Rourke et al.).

In clinical practice, arterial stiffness is measured by determination of pulse wave velocity (PWV). As the stiffness of an artery increases, the sphygmic wave velocity increases. Although different pulse wave recording techniques have been validated, applanation tonometry is the simplest and most reproducible method in clinical practice. The sphygmic wave obtained transcutaneously, by gentle compression of a superficial artery against the underlying bone plane, makes it possible to reproduce a wave whose shape is virtually identical to that recorded with an intra-arterial transducer. Measurement of PWV requires recording the pulse wave at two different locations in the arterial tree (usually the carotid and femoral artery). The recordings are synchronized with the R wave of an ECG recording made at the same time, allowing the transit time to be measured. PWV is then calculated as the path length (distance between recording sites) divided by the transit time and is expressed in m/sec (Wilkinson et al.).

Augmentation index (Aix), a surrogate for wave reflection, is another indirect and noninvasive measure of arterial stiffness. It represents the ratio of augmentation pressure (AP), which is the difference between the amplitude of the reflected wave and the amplitude of the incident wave, to PP, according to the formula AP/PP X100. The Aix is calculated by applanation tonometry; capturing the pulse wave at the radial artery or carotid artery allows the pulse wave to be derived in aorta using a transfer function.

Although both represent indirect indices of arterial stiffness, PWV and AIx are not interchangeable with each other. While PWV depends mainly on age, BP, and HR, height, sex, and HR are the main determinants of AIx. Data from the Atherosclerosis Risk in Young Adults Study (ARYA), covering a cohort of 330 young males with an average age of 28 years, report a direct relationship between AIx and PAM and an inverse relationship between AIx and height, age, and CF (Van Trijp et al.). Height partly explains why AIx is persistently higher in females than in males. The greater the height, the later the reflection of the pulse wave occurs, the point of reflection being farther from the heart. This discrepancy, however, persists even after adjustment for height, demonstrating the need for further evaluation (Janner et al.). Several studies in the last decade have investigated the effects of aging on PWV and AIx.

In the Anglo-Cardiff Collaborative Trial (ACCT), 4001 healthy subjects aged 18 to 90 years were studied. All hemodynamic indices assessed (PP, AP, AIx and PWV) significantly increased with advancing age. AIx and PWV, however, did not change linearly. In young subjects (<50 years old), AIx increased more steeply than PWV; in contrast, with aging, PWV increased more markedly than AIx (McEniery et al.). These data, confirmed by other clinical studies (Mitchell et al.) (Fantin et al.), suggest a greater sensitivity and reliability of AIx and PWV as markers of arterial stiffness in young age and adulthood, respectively. This trend is explained by the fact that in the young, the increase in AP is related to the greater amplitude of the reflected wave, rather

than to the increase in incident wave velocity. In contrast, with aging, AP increases because of the earlier return of the reflected wave and lower aortic compliance, rather than because of greater amplitude of the reflected wave.

Like PP, arterial stiffness is also a valid marker of cardiovascular (CV) risk. Indeed, numerous studies have demonstrated the positive predictive value of PWV and AIx on total and CV mortality, coronary artery disease, and stroke. In a recent meta-analysis of 17635 participants, the 1 m/s increase in PWV was associated with a 7% increase in CV risk at age 60 years. This relationship was stronger in younger subjects and was independent of sex, cigarette smoking, and the presence of hypertension, diabetes, or renal disease (Fantin et al.). The use of arterial stiffness as a biomarker of CV risk alongside traditional factors could allow for a better framing of those patients at intermediate CV risk, for whom the implementation of valid strategies aimed at event prevention is still possible and for whom a more restricted follow-up is necessary.

AIM OF THE STUDY

The aim of the study was to investigate the prevalence of cardiovascular risk factors and the presence of clinical and subclinical atherosclerotic cardiovascular disease in a single center cohort of patients with common variable immunodeficiency.

METHODS

POPULATION AND STUDY DESIGN

This is an observational retrospective-perspective study involving patients with a confirmed diagnosis of common variable immunodeficiency referring to Rare Immunological Disease Center in Cà Foncello Hospital, Treviso, Italy from January 2012 to September 2022. The diagnosis of CVID was made following the European Society for Immunodeficiencies criteria (Bonilla et al.) that include male or female patient who has a marked decrease of IgG (at least 2 SD below the mean for age) and a marked decrease in at least one of the isotypes IgM or IgA, and met all the following criteria: onset of immunodeficiency at greater than 2 years of age; absent isohemagglutinins and/or poor response to vaccines; other defined causes of hypogammaglobulinemia have been excluded
Inclusion criteria were diagnosis of CVID according to above mentioned criteria and signature of written informed consent. There were no applied exclusion criteria.

For each patient following anamnestic information were recorded: history of splenomegaly, hepatomegaly, splenectomy, cancer and cancer type, GLILD, bronchiectasis, idiopathic thrombocytopenia, autoimmunity and all other immunological comorbidities; previous cardiovascular events, metabolic syndrome (according to (Alberti et al.)), obesity, diabetes, hypertension, vasculopathies; antihypertensive medications, lipid-lowering therapy, immunosuppressive medications, immunoglobulin replace therapy; IgG, IgA and IgM titer at diagnosis.
Starting from October 2020, each patient also underwent fasting blood sampling for and routine tests such as white blood cells count, kidney function, glucose test, lipid profile, lymphocyte subpopulation, immunoglobulin levels and, in case of an adequate number of circulating B cells, gene expression analysis on B cells. To avoid any kind of bias in evaluating B cell population and cardiovascular damage, blood samples were collected apart 6 months after pregnancy or after the end of any treatment potentially impacting on B cell count and/or function.

All patients were in active follow-up at the Rare Immunological Disease Center for immunodeficiency and, if needed, at the Hypertension Clinic for cardiovascular evaluation. The study was approved by the local ethics committee (818/CE-Marca) and all enrolled subjects gave their written informed consent to participate.

VASCULAR DAMAGE EVALUATION

After blood sample, vascular damage evaluation has been performed in each patient as described below. Assessment of vascular damage was performed evaluating pulse wave velocity (PWV) and pulse wave analysis (PWA). Pulse wave velocity (PWV) is commonly used as an effective noninvasive indicator for assessing arterial stiffness, in fact, an increase in PWV is associated with large-artery stiffness, a marker of vascular damage and predicts the risk of CV event. PWV (expressed in m/s) is measured as the ratio of the distance between two measuring sites and the time delay of the arterial pulse between these sites. PWV has been measured in each patient by using a carotid tonometer simultaneously with a leg cuff to capture blood pressure waveforms at the carotid and femoral sites. Physical distance measurements allowed the software to perform the velocity calculation.

PWA was performed to record the brachial artery waveforms and generate a corresponding central (ascending aortic) waveform. From this, central (aortic) BP, measures of arterial wave reflections (augmentation index [AIx] and augmentation pressure [AP]), mean arterial pressure (MAP), heart rate (HR), and pulse pressure (PP) amplification has been obtained from each patient.

PWV and PWA were performed using non-invasive pulse wave analysis monitoring system (SphygmoCor Xcel - AtCor Medical, Australia).

Carotid ultrasound examinations were performed to establish the presence of carotid plaques and measure IMT. IMT has been measured following a standardized protocol according to the Mannheim Intima-Media Thickness Consensus. Briefly, the right and left carotid arteries of each subject has been examined by the same sonographer. IMT, defined as the distance between the lumen-intima and the media-adventitia interfaces (expressed in mm), has been measured at end diastole in the far wall of the right and left sides of the common carotid artery, the bulb and the internal carotid artery. IMT measurements has been expressed as cumulative mean of mean-IMT (mean-IMT) and as cumulative mean of maximum-IMT (M-MAX) recorded in each vascular segment. The presence of plaques has been identified as focal structures encroaching into the arterial lumen of at least 0.5 mm or 50% of the surrounding IMT value, or demonstrating a thickness >1.5 mm as measured from the intima-lumen interface to the media-adventitia interface.

T AND B CELLS IMMUNOPHENOTYPING

White blood cells were identified using flow cytometry. Flow-cytometric analysis has been performed in hematology laboratory according to standardize diagnostic protocols, by FACSCanto II (BD Biosciences™). For each patient immunophenotype and B cell subpopulations were identified following the standard defined by the EUROClass trial as follow: Naïve IgD+IgM+CD27−; Switched memory IgD−IgM−CD27+; Marginal zone IgD+IgM+CD27+; Transitional CD38++IgM^{high}; Activated CD21^{low}CD38^{low}; Plasmablasts CD38+++IgM− (Wehr et al.). Monoclonal antibodies were conjugated as follow:

- **Immunophenotype**:
    - **CD4** – *FITCH/Alexa488*
    - **CD8** – *PE*
    - **DR** – *PerCP/PerCP-PE-CY5*
    - **CD5** – *PE-CY7*
    - **CD19** – *APC/Alexa647*
    - **CD45** – *APC-CY7*

    - **CD57** – *FITCH/Alexa488*
    - **GD** – *PE*
    - **CD16** – *PerCP/PerCP-PE-CY5*
    - **CD56** – *PE-CY7*
    - **CD3** – *APC/Alexa647*
    - **CD45** – *APC-CY7*

    - **CD45** – *APC-CY7*

- **EUROClass**:
    - **IgM** – *FITCH/Alexa488*
    - **IgD** – *PE*
    - **CD38** – *Per-CP/PerCP-PE-CY5*
    - **CD21** – *PE-CY7*
    - **CD27** – *APC/Alexa647*
    - **CD19** – *APC-CY7/APC-H7*

IMMUNOMAGNETIC ISOLATION OF CD19+ B CELLS

Peripheral blood mononuclear cells (PBMC) from CVID were isolated from blood sample using SepMate tubes (Stemcell Technologies™) according to the manufacturer's protocol. CD19+ B

cells were isolated from PBMC using Dynabeads Untouched Human B Cells kit (Invitrogen™). Briefly, not more than 50MLN of PBMC were incubated with 100uL of antibodies for a negative selection of CD19+ B cells. After incubation 500uL of Dynabeads were added, tubes were placed in the magnet and supernatant containing the untouched B cells taken.

To access the purity of isolated B cells suspension, cells were incubated with CD19, CD3, CD16, CD123 antibody and tested using cytofluorimeter. The strength of this immunomagnetic isolation is to provide, with a highly sensitive method, untouched B cells that are suitable for gene expression experiments. However, this technique presents a low yield that could be a problem when processing samples with low number of B cells.

LDLr AND MR GENE EXPRESSION

B cells total RNA was isolated using RNeasy Plus Mini Kit or RNeasy Plus Micro Kit (Quiagen™) following manufacturer's protocol. The quantification and the quality were determined by spectrophotometric readings at 260/280/230 nm using a Nanodrop (Thermo).

The retrotranscription was performed on 100 ng of total RNA in a final volume of 20 uL using the iScript™ cDNA Synthesis kit (Bio-Rad™, Milan, Italy) following the manufacturer's recommendations.

Gene expression analysis was performed by Droplet Digital™ PCR (ddPCR™) technique which provides ultrasensitive nucleic acid detection and absolute quantification. It is highly effective for resolving low abundance targets

The ddPCR was performed on 25 ng of cDNA in a total volume of 20 µL reaction mixture, containing 10 µL EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA), each primer set (final concentration, 200 nM), DNase/RNase-free sterile water. The sequences of each primer are listed in Supplementary Table S1. Each ddPCR mixture plus 70 µL of generation oil for EvaGreen was loaded into each sample and transferred to a 96-well PCR plate using the Automated Droplet Generator (Bio-Rad™, Milan, Italy). The PCR plate was placed in a C1000 thermal cycler with a deep well reaction module (Bio-Rad Laboratories) for amplification. Thermal cycling conditions were as follows: 5 minutes at 95℃, followed by 40 cycles of denaturation for 30 seconds at 95℃, annealing/extension for 1 minute at 58℃, and three steps at 4℃ for 5 minutes, 90℃ for 5 minutes, and a 4℃ infinite hold. After thermal cycling, the PCR plate containing the droplets was loaded onto the QX200 droplet reader (Bio-Rad Laboratories) to measure the fluorescence intensity of the

EvaGreen fluorophore within each droplet using a multipixel photon counter. The detector reads the droplets and identifies those that contain a target gene (+) and those that do not (−). It then plots the fluorescence droplet-by-droplet. The QuantaSoft software (version 1.7.4, Bio-Rad Laboratories) was used to calculate the concentration (copies/µL) of the target genes. Results are expressed as copies/uL of target normalized for copies/uL of housekeeping (GAPDH).

CLINICAL PHENOTYPE CLASSIFICATION

CVID patients were classified according to *Chapel et al.* by differentiating clinical phenotype (Chapel et al.). The clinical phenotypes were defined as follows:

1. CHAPEL 1: no disease-related complications (described as ‘‘infections only’’)
2. CHAPEL 2: autoimmunity (including organ-specific autoimmune diseases and cytopenia)
3. CHAPEL 3: polyclonal lymphoproliferation (including hepatomegaly, persistent unexplained lymphadenopathy, lymphoid interstitial pneumonitis, noninfective granuloma)
4. CHAPEL 4: unexplained enteropathy (excluding infective, autoimmune, and gluten-sensitive enteropathies)

CKD was defined according to KDIGO 2012 clinical practice guideline for the evaluation and management of chronic kidney disease (Levin et al.). Dyslipidemia was defined according to 2019 ESC/EAS Guidelines if LDL cholesterol was > 116 mg/dL (Mach et al.). Metabolic syndrome was defined according to the harmonized criteria (Alberti et al.). Clinical diagnosis of metabolic syndrome was done if patient met at least three of the following criteria:

1. Triglycerides > 150 mg/dL or lipid lowering treatment;
2. HDL cholesterol < 40 mg/dL in male, < 50 mg/dL in female;
3. Hypertension (defined as systolic > 130 mmHg, diastolic > 85mmHg) or drug treatment with a history of hypertension;
4. Fasting glucose > 100 mg/dL or drug treatment for elevated glucose;
5. Elevated waist circumference population and country specific (> 94 cm in male, > 80 cm in female);

STATISTICS

Statistical analysis was performed using SPS Statistics version 28.0.1 (IBM™ New York, US). Continuous variable data were expressed as mean ± standard deviation if normally distributed or median ± standard deviation if not. Categorical data were expressed like number of subjects and percentage of cases on present data. Comparisons between groups of continuous variables were assessed using a parametric ANOVA or a non-parametric Kruskal–Wallis test, according to the results of the Shapiro–Wilk test of normality. Fisher exact test was used to compare two categorical variables, Pearson's χ^2 test was used to compare more than two categorical variables. PWV comparison between groups was performed after adjustment for HR and MAP while AP and AIx comparisons were performed after adjustment for HR and body height. p value less than 0.05 was considered significant.

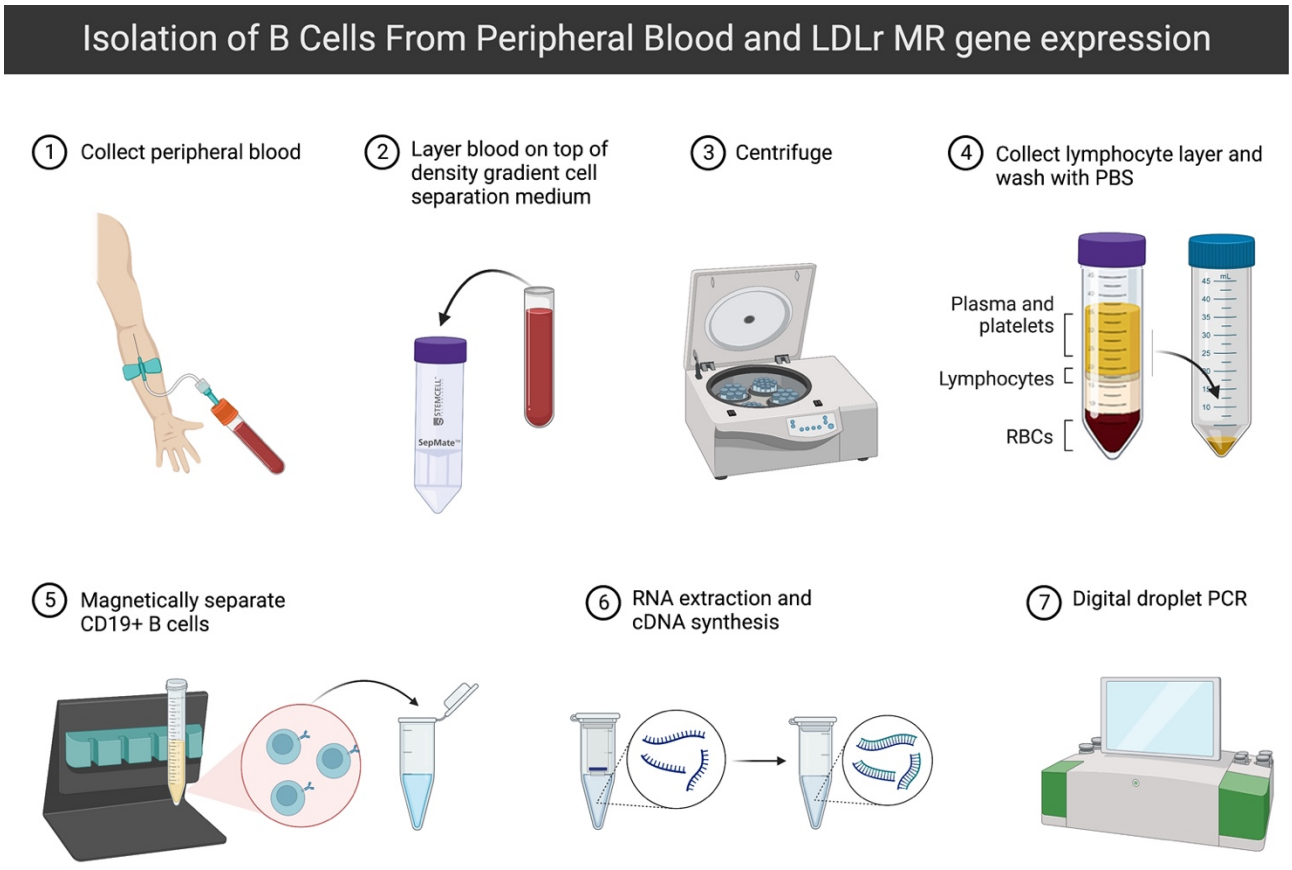


Figure 2 Flow chart of B cells isolation and gene expression analysis process.

RESULTS

GENERAL CHARACTERISTICS OF COHORT

A total of 127 patients affected by CVID were included in the study. General characteristics of study population are reported in Table 1. Mean age was 51.6 years and patients were equally distributed between male and female (44.1% male, 55.9% female). Mean BMI was 24.2 $\pm$ 5.1 kg/m^2 while SBP was 127 $\pm$ 14.9 mmHg and DBP was 76 $\pm$ 8.3 mmHg. Hypertension was found in 40 patients (34.2%), all of them were taking chronic antihypertensive medications.

23 patients, accounting for 20% of the entire population, showed previous history of dyslipidemia, of which 18 patients treated with lipid-lowering medications. However, after blood sample analysis, more than fifty percent of patients showed a hypercholesterolemic condition defined according to 2019 ESC/EAS Guidelines (Mach et al.) (total cholesterol: 194 $\pm$ 44.2 mg/dL, LDL-cholesterol: 122 $\pm$ 40.2, HDL-cholesterol: 52 $\pm$ 14.5). 23.7% of patients presented a previous diagnosis of metabolic syndrome. History of diabetes was found in 15 patients; 13 patients were taking anti-diabetes therapy while 2 patients were in diet restriction (Table 1).

GENERAL CHARACTERISTICS	N (127)
Age, years (SD)	51.6 (14.3)
Gender – male, n (%)	56 (44.1)
BMI, kg/m^2 (SD)	24.2 (5.1)
Waist circumference, cm (SD)	94.4 (14.3)
SBP, mmHg (SD)	127 (14.9)
DBP, mmHg (SD)	76 (8.3)
Total cholesterol, mg/dL (SD)	194 (44.2)
LDL-cholesterol, mg/dL (SD)	122 (40.5)
HDL-cholesterol, mg/dL (SD)	52 (14.5)
Tryglicerides, mg/dL (SD)	102 (74.5)
Glycemia, mg/dL (SD)	94 (27.8)
HbA1c, mmol/mol (SD)	37 (10.1)
Creatinine, mg/dL (SD)	0.7 (0.2)
eGFR, mL/min/1.73m^2 (SD)	96.5 (22.3)
Microalbuminuria, mg/dL (SD)	3.7 (8.1)
Serum sodium, mEq/L (SD)	140 (3.3)
CRP, mg/dL (SD)	2.7 (4.5)

RISK FACTORS AND ONGOING DRUG THERAPY	N (127)
Hypertension, n (%)	40 (34.2)
Hypertension therapy, n (%)	40 (34.2)
History of dyslipidemia, n. (%)	23 (20.0)
Hypercholesterolemia at first visit, n. (%)	58 (51.3)
Lipid-lowering therapy, n (%)	18 (15.7)
History of metabolic syndrome, n. (%)	27 (23.7)
Diabetes, n (%)	15 (13.3)
Diabetes therapy, n (%)	13 (11.4)
Glucocorticoids therapy, n (%)	23 (19.7)
Antinflammatory drugs, n (%)	3 (2.6)
Antiplatelet therapy, n (%)	10 (8.5)
Antibiotic prophylaxis, n (%)	13 (11.0)
Immunosuppressive treatment, n (%)	12 (10.2)

Table 1 General characteristics, risk factors and drug therapies of patients. Data are expressed as means ± standard deviation (SD) or number of patients and percentage (%). BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low density lipoprotein; HDL, high density lipoprotein; CRP, C-reactive protein.

Anamnestic cardiovascular events and immunodeficiency-related symptoms are reported in Table 2. Previous cardiovascular events (that include MI, stroke/TIA, VTE) occurred in 9 patients, accounting for 7.1% of the overall population included in the study. The more frequent cardiovascular events were stroke/TIA and venous thromboembolism (VTE), only two patients had history of ACS. As for CVID diagnosis, the mean age of symptoms onset was 31 years while a confirmed diagnosis had been achieved 10 years later. The most frequent immunodeficiency-related complications were bronchiectasis found in 36.1%, and autoimmunity, accounting for 33.9% of cases, while GLILD occurred in 19.2% of patients (Table 2).

HISTORY OF CARDIOVASCULAR EVENTS AND CVID CLINICAL FEATURES	N (127)
Previous CV events, n (%)	9 (7.1)
Smoke – active, n (%)	15 (12.5)
History of ACS, n (%)	2 (1.7)
History of stroke/TIA, n (%)	4 (3.4)
History of PAD, n (%)	1 (0.8)
History of VTE, n (%)	4 (3.4)
Carotid plaque, n (%)	5 (4.5)
LVH, n. (%)	7 (5.9)
CKD, n. (%)	3 (2.6)
Age symptoms onset, years (SD)	31.0 (16.6)
Age CVID diagnosis, years (SD)	41.3 (15.3)
Splenomegaly, n (%)	43 (36.4)
Splenectomy, n (%)	8 (6.8)
Hepatomegaly, n (%)	8 (6.8)
Cancer, n (%)	26 (22.0)
GLILD, n (%)	23 (19.2)
Bronchiectasis, n (%)	43 (36.1)
Autoimmunity, n (%)	40 (33.9)
ITP, n (%)	15 (12.7)

Table 2 Previous cardiovascular events and disease related complications of CVID patients. Data are expressed as means ± standard deviation (SD) or number of patients and percentage (%). ACS, acute coronary syndrome; TIA, transitory ischemic event; PAD, peripheral artery disease; VTE, venous thromboembolism; LVH, left ventricular hypertrophy; CKD; chronic kidney disease; CVID, common variable immunodeficiency; GLILD, Granulomatous Lymphocytic Interstitial Lung Disease; ITP, autoimmune thrombocytopenia.

White blood cells and differential T and B cells count are reported in Table 3. CVID patients showed a 32.1% of lymphocytes, 76.6% of CD3+, 42.5% of CD4+, 35.0% of CD8+ and 7.5% of CD19+ cells. B cell subsets showed a decrease in all subtypes of CD19+ cells (B naïve CD27-IgM+IgD+: 70.7%, B marginal CD27+IgM+IgD+: 16.0%, B switched memory CD27+IgM-IgD-: 7.0%, B transitional

CD38++IgM++: 1.6%, B activated $CD21^{low}CD38^{low}$: 7.6%, B plasmablasts CD38++IgM-: 0.4%). According to the specific characteristics of CVID disease, immunoglobulin levels at diagnosis showed a severe reduction in IgG (341 $\pm$ 162 mg/dL), IgA (23 $\pm$ 40.5 mg/dL) and IgM (27 $\pm$ 139 mg/dL) (Table 4). Immunoglobulin steady-state level has been achieved treating 93% of patients with immunoglobulin replace therapy starting at 334 $\pm$ 151.1 mg/kg/4 weeks to raise a mean final dose of 371 $\pm$ 162.6 mg/kg/4 weeks (Table 4).

IMMUNOLOGICAL FEATURES	N (127)
Lymphocytes, % (SD)	32.1 (10.5)
CD3, % (SD)	76.6 (11.6)
CD4, % (SD)	42.5 (12.7)
CD8, % (SD)	35.0 (11.9)
CD4/CD8, % (SD)	1.4 (0.8)
CD3+CD8+CD57+, % (SD)	18.1 (13.2)
CD19, % (SD)	7.5 (6.4)
B naïve CD27-IgM+IgD+, % (SD)	70.7 (19.3)
B marginal CD27+IgM+IgD+, % (SD)	16.0 (15.0)
B switched memory CD27+IgM-IgD-, % (SD)	7.0 (7.2)
B transitional CD38++IgM++, % (SD)	1.6 (3.5)
B plasmablasts CD38++IgM-, % (SD)	0.4 (1.1)
B activated $CD21^{low}CD38^{low}$, % (SD)	7.6 (8.7)

Table 3 Immunological features of CVID patients. Data are expressed as means ± standard deviation (SD).

IMMUNOGLOBULINE REPLACE THERAPY	N (127)
IgG diagnosis, mg/dL (SD)	341 (178.6)
IgA diagnosis, mg/dL (SD)	23 (40.5)
IgM diagnosis, mg/dL (SD)	27 (139.9)
IGRT, n (%)	107 (93)
IGRT initial dose, mg/kg/4week (SD)	334 (152.1)
IGRT steady state, mg/kg/4week (SD)	371 (162.6)
IgG steady state, mg/dL (SD)	788 (249.5)
IgA steady state, mg/dL (SD)	37 (73.7)
IgM steady state, mg/dL (SD)	61 (160.6)

Table 4 Immunoglobulin levels and replace therapy in CVID patients. Data are expressed as means ± standard deviation (SD) or number of patients and percentage (%). IGRT, immunoglobulin replace therapy.

INFECTION ONLY vs COMPLICATED

The first distinction between those patients is based on clinical phenotype. In particular, during the disease progression, patients can be classified into two main groups: those presenting symptoms only due to recurrent infections and those presenting more complications like autoimmunity, limphoprolipherative disorders, granulomatous and lymphocytic interstitial lung disease, cancer or enteropathy. Therefore, patients have been classified as "infections only" (58.7%) or "complicated phenotype" (41.3%) based on the clinical features (Figure 3).

Table 5 present the comparison between the two groups. Patients with the complicated phenotype showed similar age (49.2 ± 14.4 years versus 53.2 ± 14.1 years) and BMI (24.7 ± 4.5 kg/m^2 versus 25.7 ± 5.5 kg/m^2) to those with only infections.

Also, SBP (125 ± 14.1 mmHg versus 129 ± 15.2) and DBP (75 ± 8.4 mmHg versus 77 ± 8.2 mmHg) were similar while hypertension prevalence showed a slight increase in patients whit a complicated phenotype (36.7% versus 29.9%) even if not significant.

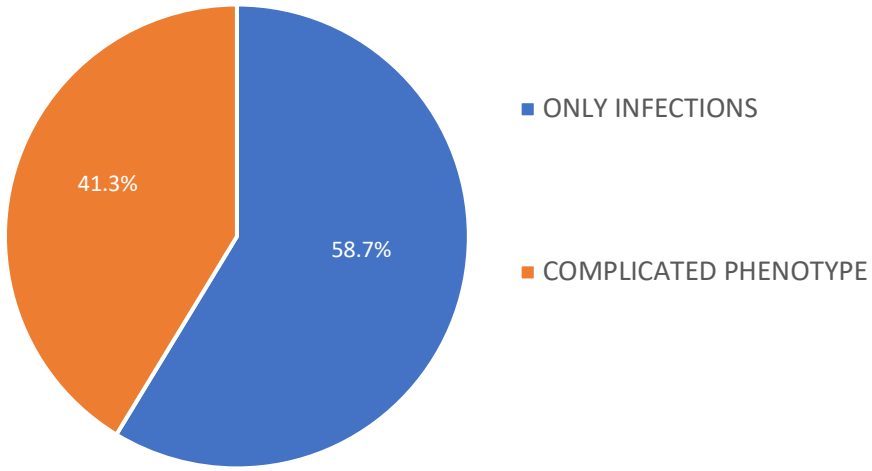


Figure 3 Distribution of CVID patients presenting only infections or a complicated phenotype. Data are expressed as percentage of the entire cohort.

	ONLY INFECTION	COMPLICATED PHENOTYPE	p value
N.	74	52	
Age, years (SD)	53.2 (14.1)	49.2 (14.5)	0.111
Gender – male, n (%)	31 (42.5)	24 (46.2)	0.524
BMI, kg/m^2 (SD)	25.7 (5.5)	24.7 (4.5)	0.402
Waist circumference, cm (SD)	94.4 (13.4)	94.4 (15.6)	0.774
SBP, mmHg (SD)	129 (15.2)	125 (14.1)	0.192
DBP, mmHg (SD)	77 (8.2)	75 (8.4)	0.420
Total cholesterol, mg/dL (SD)	206 (44.5)	179 (40.1)	**0.001**
LDL-cholesterol, mg/dL (SD)	131 (42.2)	112 (36.4)	**0.025**
HDL-cholesterol, mg/dL (SD)	56 (14.6)	48 (13.4)	**0.003**
Triglycerides, mg/dL (SD)	112 (55.8)	130 (92.4)	0.486
Glycemia, mg/dL (SD)	98 (17.6)	88 (14.9)	**0.006**
HbA1c, mmol/mol (SD)	38 (6.0)	33 (4.5)	**<0.001**
Creatinine, mg/dL (SD)	0.8 (0.2)	0.8 (0.2)	0.828
eGFR, mL/min/1.73m^2 (SD)	90 (18.0)	93 (28.0)	0.187
Microalbuminuria, mg/dL (SD)	8.5 (9.3)	3.9 (5.1)	**0.032**
Serum sodium, mEq/L (SD)	138 (3.6)	138 (3.2)	0.862
CRP, mg/dL (SD)	3.9 (4.4)	3.5 (4.6)	0.362

Table 5 Comparison of general characteristics and humoral biochemical data between CVID patients presenting only infections and those with complicated phenotype. Data are expressed as means ± standard deviation (SD). Data are expressed as means ± standard deviation (SD) or number of patients and percentage (%). BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low density lipoprotein; HDL, high density lipoprotein; CRP, C-reactive protein.

Lipid profile of patients showed a significant different level in total cholesterol (179 ± 40.1 mg/dL versus 206 ± 44.5 mg/dL, p=0.001), LDL-cholesterol (112 ± 36.4 mg/dL versus 131 ± 42.2 mg/dL, p=0.025) and HDL-cholesterol (48 ± 13.4 mg/dL versus 56 ± 14.6 mg/dL, p=0.003) (Table 5 and Figure 4) that were lower in patients with the complicated phenotype even if the lipid-lowering treatment were the same in both groups (14.9% versus 16.4%, p = 0.543) (Table 6). The values reported in lipid profile were calculated excluding patients under lipid-lowering treatment. However, even if including those patients, the p value was still significant (data not shown).

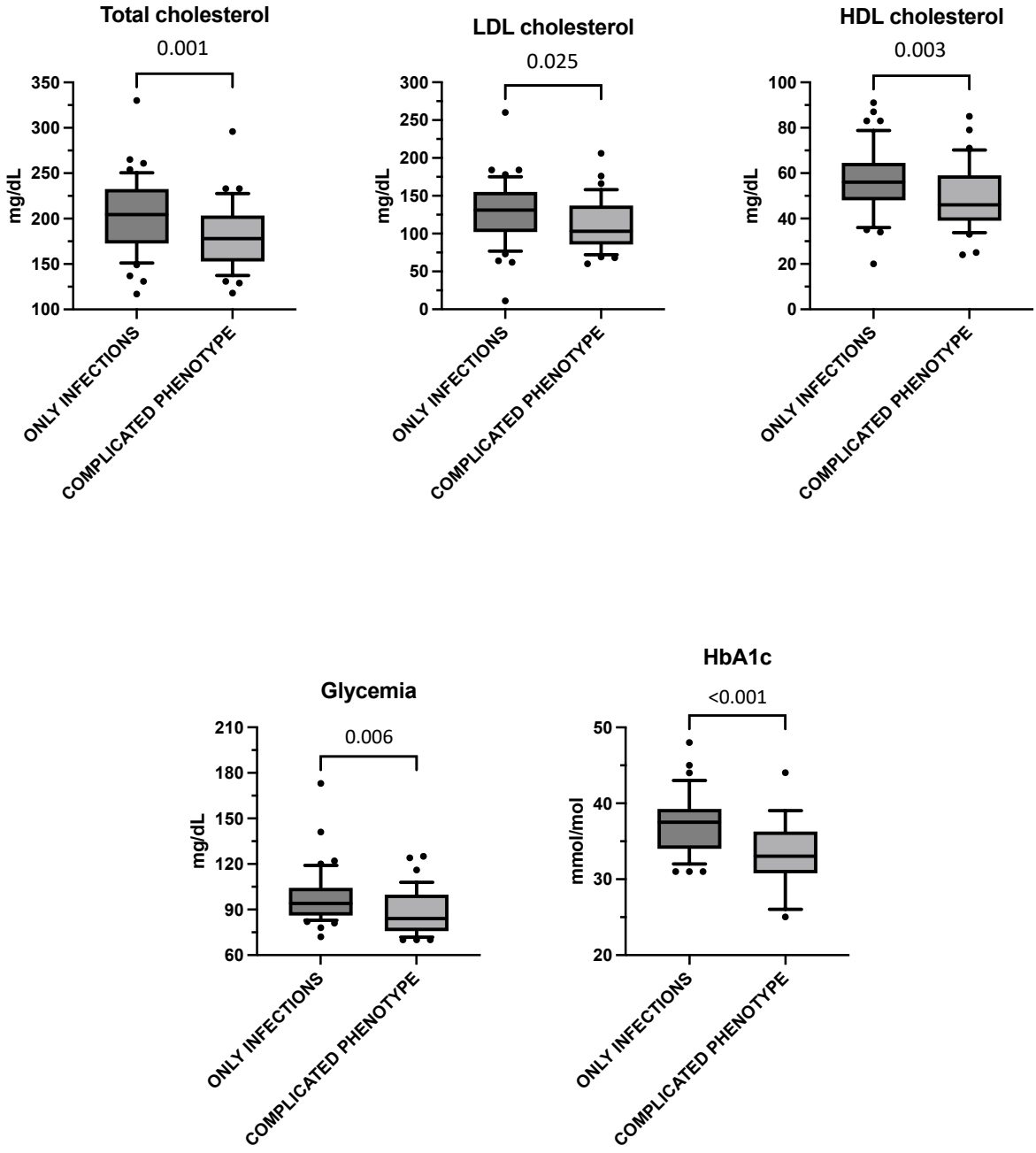


Figure 4 Comparison of lipid profile, glycemia and HbA1c between CVID patients presenting only infections and those with a complicated phenotype. Data are expressed as means ± standard deviation (SD).

	ONLY INFECTION	COMPLICATED PHENOTYPE	p value
N.	74	52	
Hypertension, n (%)	21 (31.3)	18 (36.7)	0.332
Hypertension therapy, n (%)	20 (29.9)	18 (36.7)	0.204
History of dyslipidemia, n. (%)	14 (20.6)	9 (19.1)	0.560
Hypercholesterolemia at first visit, n. (%)	37 (57.8)	21 (42.9)	0.083
Lipid-lowering therapy, n (%)	11 (16.4)	7 (14.9)	0.543
History of metabolic syndrome, n. (%)	13 (19.4)	14 (30.4)	0.121
Metabolic syndrome at first visit, n. (%)	18 (26.9)	20 (40.0)	0.097
Diabetes, n (%)	8 (12.5)	7 (14.6)	0.563
Diabetes therapy, n (%)	6 (9.2)	7 (14.6)	0.278
Glucocorticoids therapy, n (%)	8 (11.6)	14 (29.8)	**0.014**
Antinflammatory drugs, n (%)	2 (2.9)	1 (2.1)	0.659
Antiplatelet therapy, n (%)	5 (7.2)	5 (10.6)	0.345
Antibiotic prophylaxis, n (%)	6 (8.8)	6 (12.2)	0.556
Immunosuppressive treatment, n (%)	0 (0.0)	11 (22.4)	**<0.001**

Table 6 Comparison of drug therapy and risk factors in CVID patients presenting only infections and those with complicated phenotype. Data are expressed as number of patients and percentage (%).

Diabetes prevalence was the same in both groups (14.6% versus 12.5%) as the diabetes therapy (Table 6). However, patients presenting complicated phenotype showed significantly lower glycemia (88 $\pm$ 14.9 mg/dL versus 98 $\pm$ 17.6 mg/dL, p=0.006) and HbA1c levels (33 $\pm$ 4.5 mmol/mol versus 36 $\pm$ 6.0 mmol/mol, p<0.001) (Table 5 and Figure 4), even if in the complicated phenotype group, the number of patients under treatment with glucocorticoids was higher (29.8% versus 11.6%, p=0.014). The values reported in glycemia and HbA1c were calculated excluding patients under diabetes therapy. However, even if including those patients, the p value was still significant (data not showed).

Even if the history of chronic kidney disease (CKD) was similar between groups (3.3% versus 1.4%) (Table 7), microalbuminuria levels were significantly lower in patients presenting complicated phenotype (3.9 $\pm$ 5.1 mg/dL versus 8.5 $\pm$ 9.3 mg/dL p=0.032) (Table 5).

	ONLY INFECTION	COMPLICATED PHENOTYPE	p value
N.	74	52	
Previous CV events, n (%)	5 (7.2)	4 (8.2)	0.524
Smoke – active, n (%)	11 (15.7)	4 (8.2)	0.169
History of ACS, n (%)	1 (1.4)	1 (2.2)	0.652
History of stroke/TIA, n (%)	3 (4.3)	1 (2.2)	0.468
History of PAD, n (%)	1 (1.4)	0 (0.0)	0.597
History of VTE, n (%)	2 (2.9)	2 (4.3)	0.514
Carotid plaque, n (%)	4 (6.2)	1 (2.2)	0.316
LVH, n. (%)	5 (7.2)	2 (4.3)	0.420
CKD, n. (%)	1 (1.4)	2 (4.3)	0.341
Age symptoms onset, years (SD)	33.5 (16.6)	28.0 (16.3)	0.105
Age diagnosis, years (SD)	42.6 (15.6)	39.2 (14.8)	0.299
Splenomegaly, n (%)	18 (26.9)	24 (48.0)	**0.015**
Splenectomy, n (%)	0 (0.0)	8 (16.0)	**<0.001**
Hepatomegaly, n (%)	4 (6.2)	4 (8.2)	0.551
Cancer, n (%)	17 (25.4)	9 (18.0)	0.150
GLILD, n (%)	1 (1.4)	22 (43.1)	**<0.001**
Bronchiectasis, n (%)	22 (32.4)	21 (42.0)	0.189
Autoimmunity, n (%)	15 (22.1)	24 (49.0)	**0.007**
ITP, n (%)	1 (1.4)	14 (28.6)	**<0.001**

Table 7 Comparison of previous cardiovascular events and disease related complications of patients presenting only infections and those with a complicated phenotype. Data are expressed as means ± standard deviation (SD) or number of patients and percentage (%). ACS, acute coronary syndrome; TIA, transitory ischemic event; PAD, peripheral artery disease; VTE, venous thromboembolism; LVH, left ventricular hypertrophy; CKD; chronic kidney disease; CVID, common variable immunodeficiency; GLILD, Granulomatous Lymphocytic Interstitial Lung Disease; ITP, autoimmune thrombocytopenia.

According to the clinical classification of patients, it was not surprising that the frequence of complications related to disease progression like splenomegaly, splenectomy, GLILD, autoimmunity and ITP, was significantly higher in the complicated phenotype group compared to patients presenting only infections (Table 7).

	ONLY INFECTION	COMPLICATED PHENOTYPE	p value
N.	74	52	
Lymphocytes, % (SD)	32.4 (10.5)	31.6 (10.5)	0.628
CD3, % (SD)	74.0 (12.1)	79.1 (10.4)	**0.048**
CD4, % (SD)	42.7 (13.6)	42.2 (11.2)	0.997
CD8, % (SD)	33.4 (11.2)	37.5 (12.7)	0.184
CD4/CD8, % (SD)	1.4 (0.9)	1.3 (0.7)	0.854
CD3+CD8+CD57+, % (SD)	14.5 (11.7)	23.5 (13.7)	**<0.001**
CD19, % (SD)	7.5 (6.0)	7.5 (6.9)	0.635
B naïve CD27-IgM+IgD+, % (SD)	68.5 (20.7)	74.1 (16.7)	0.213
B marginal CD27+IgM+IgD+, % (SD)	17.8 (16.0)	13.2 (13.1)	0.066
B switched memory CD27+IgM-IgD-, % (SD)	8.4 (8.1)	4.7 (4.9)	**0.005**
B transitional CD38++IgM++, % (SD)	0.8 (0.9)	2.7 (5.4)	0.056
B plasmablasts CD38++IgM-, % (SD)	0.4 (1.0)	0.4 (1.2)	0.173
B activated $CD21^{low}CD38^{low}$, % (SD)	6.8 (8.3)	8.9 (9.2)	0.224

Table 8 Comparison of immunological features of CVID patients presenting only infections and those with a complicated phenotype. Data are expressed as means ± standard deviation (SD).

With regards to immune cells analysis (Table 8), patients with complicated phenotype presented higher levels of CD3+ cells (79.1% versus 74.0%, p=0.048) and CD3+CD8+CD57+ cells (23.5% versus 14.5%, p<0.001) compared to patients presenting only infections. Also, switched memory B cells (CD27+IgM-IgD-) were lower in patients with complicated phenotype (4.7% versus 8.4%, p=0.005). At diagnosis those patients showed significantly lower levels of IgG (284 $\pm$ 159.4 mg/dL versus 380 $\pm$ 182.0 mg/dL, p=0.014), IgA (24 $\pm$ 33.6 mg/dL versus 43 $\pm$ 43.9 mg/dL, p=0.008) and IgM (39 $\pm$ 55.1 mg/dL versus 79 $\pm$ 177.9 mg/dL, p=0.033) compared to patients presenting only infections (Table 9). Consequently, IGRT (92.3% versus 87.9%, p=0.010) and the administered dose to raise immunoglobulins steady-state level (415 $\pm$ 213.5 mg/dL versus 341 $\pm$ 91.5 mg/dL, p=0.049) were significantly higher. Even after IGRT, patients with complicated phenotype showed a significantly lower level of IgA (19 $\pm$ 24.8 mg/dL versus 50 $\pm$ 91.5 mg/dL, p=0.017) (Table 9).

	ONLY INFECTION	COMPLICATED PHENOTYPE	p value
N.	74	52	
IgG diagnosis, mg/dL (SD)	380 (182.0)	284 (159.4)	**0.014**
IgA diagnosis, mg/dL (SD)	43 (43.9)	24 (33.6)	**0.008**
IgM diagnosis, mg/dL (SD)	79 (177.9)	39 (55.1)	**0.033**
IGRT, n (%)	58 (87.9)	48 (92.3)	**0.010**
IGRT initial dose, mg/kg/4week (SD)	325 (89.6)	343 (198.3)	0.840
IGRT steady state, mg/kg/4week (SD)	341 (91.5)	415 (213.5)	**0.049**
IgG steady state, mg/dL (SD)	767 (271.9)	821 (210.2)	0.158
IgA steady state, mg/dL (SD)	50 (91.5)	19 (24.8)	**0.017**
IgM steady state, mg/dL (SD)	69 (196.0)	50 (86.6)	0.348

Table 9 Comparison of immunoglobulin levels and replace therapy in CVID patients presenting only infections and those with a complicated phenotype. Data are expressed as means ± standard deviation (SD) or number of patients and percentage (%). IGRT, immunoglobulin replace therapy.

The study of vascular stiffness and IMT was performed in a subgroup of 55 consecutive patients attending the outpatient clinic, of which 33 patients presenting only infections and 22 patients with clinical complications (Table 10). PWV comparison was performed after adjustment for HR and MAP while AP and Aix comparisons were performed after adjustment for HR and body height. Even if the difference was not significant, all evaluated markers showed higher levels in group of patients with only infections. PP and AP were significantly higher in these patients, suggesting that vascular stiffness might be more present if compared with same age and sex patients with a complicated phenotype. IMT did not show differences between groups

	ONLY INFECTION	COMPLICATED PHENOTYPE	p value
N.	33	22	
Age, years (SD)	55.0 (13.5)	49.1 (13.5)	0.111
Gender – male, n (%)	9 (27.3)	10 (40.0)	0.229
BMI, kg/m^2 (SD)	26.8 (6.1)	23.7 (3.3)	**0.025**
Smoke – active, n (%)	3 (9.4)	1 (4.5)	0.587
LVH, n. (%)	3 (9.4)	0	0.224
History of metabolic syndrome, n. (%)	6 (19.4)	5 (26.3)	0.405
Diabetes, n (%)	4 (13.3)	2 (9.0)	0.544
Hypertension, n (%)	10 (31.3)	6 (27.2)	0.588
SBP, mmHg (SD)	123 (16.1)	116 (14.0)	0.093
DBP, mmHg (SD)	79 (9.5)	77 (10.0)	0.758
PP, mmHg (SD)	44 (9.6)	39 (11.9)	**0.042**
MAP, mmHg (SD)	98 (12.6)	94 (11.3)	0.290
HR, bpm (SD)	71 (12.3)	74 (16.4)	0.608
AP, mmHg (SD)	17 (7.3)	13 (7.7)	**0.033**
AIx, % (SD)	37 (12.8)	31 (12.9)	0.104
AIx 75, % (SD)	36 (13.3)	33 (13.8)	0.225
PWV, m/s (SD)	6.3 (1.2)	5.9 (1.0)	0.356
IMT carotid max, mm (SD)	0.6 (0.1)	0.5 (0.1)	0.422

Table 10 Comparison of vascular stiffness and atherosclerosis markers between CVID patients presenting only infections and those with a complicated phenotype. Data are expressed as means ± standard deviation (SD) or number of patients and percentage (%). BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure; HR, heart rate; AP, augmentation pressure; Aix, augmentation index; PWV, pulse wave velocity; IMT, intima-media tickness.

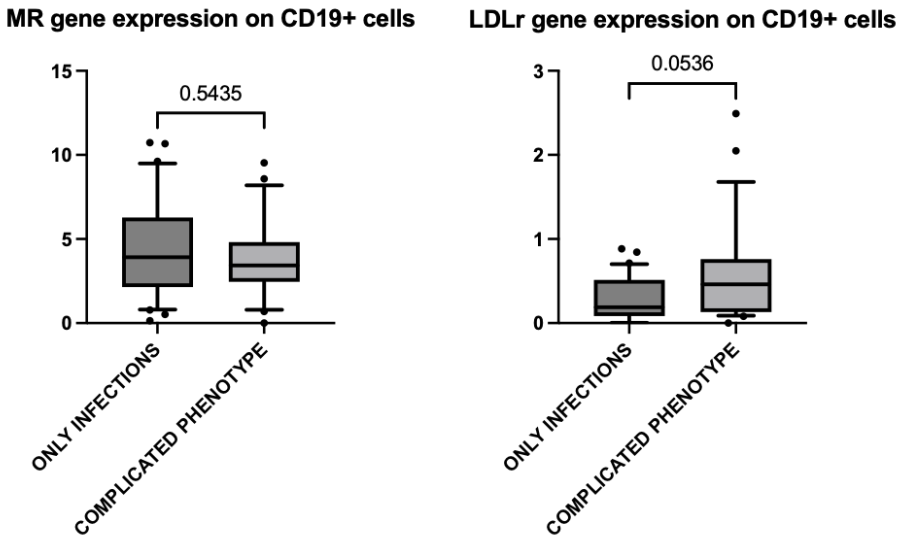


Figure 5 Comparison of LDLr and MR gene expression on CD19+ B cells in patients presenting only infections and those with a complicated phenotype. Data has been normalized on GAPDH gene expression.

To determine the role of LDLr and MR in development of cardiovascular disease, gene expression analysis was performed. Untouched total CD19+ B cells of 31 patients with only infections and 20 patients with a complicated clinical phenotype were investigated. The results showed that MR and LDLr copies/UL normalized on GAPDH copies/uL were not statistically significant different above groups, even if patients with the complicated phenotype presented higher gene expression levels of LDLr (Figure 5).

CHAPEL COMPARISON

Based on previous results and to investigate if the cardiovascular characteristics of patients correspond to a different clinical phenotype, CVID patients have been divided in four main groups according to Chapel *et al*. classification: patients presenting only infections (CHAPEL 1 = 58.7%), autoimmunity (CHAPEL 2 = 5.6%), lymphoproliferation (CHAPEL 3 =13.5%) and enteropathy (CHAPEL 4 = 8.7%) (Figure 6A). Some patients presented an intermediate/overlapping phenotype, the majority of them presented autoimmunity with lymphoproliferation (CHAPEL 2+3 = 9.5%) while some of them presented autoimmunity, lymphoproliferation and enteropathy (CHAPEL 2+3+4 = 4.0%).

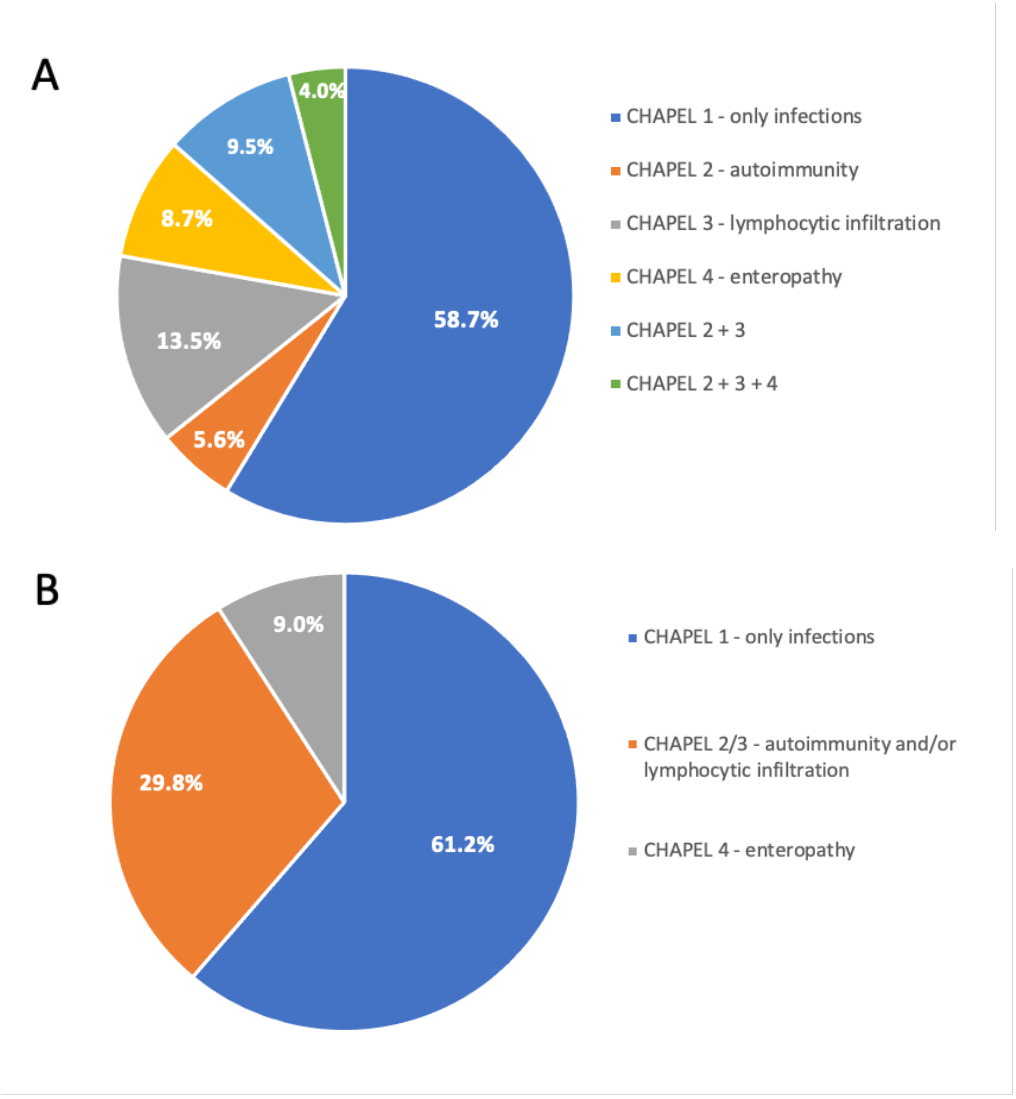


Figure 6 A) Distribution of CVID patients presenting only infections, autoimmunity, limphocytic infiltration, enteropathy or combined complications at enrollment. B) Distribution of CVID patients after analysis of clinical and biochemical characteristics, patients with autoimmunity and/or limphocytic infiltration were grouped together. Data are expressed as percentage of the entire cohort.

Of note, we observed a high level of overlap in clinical and biochemical characteristics of patients belonging to CHAPEL 2 and CHAPEL 3. For this reason and in order to point out specific characteristics depending on clinical manifestation and immunological background of patients, we decided to conduct further analysis by combining CHAPEL 2 with CHAPEL 3 and CHAPEL 2+3. Five patients presenting enteropathy associated to autoimmunity and lymphocytic infiltration were excluded from the analysis. As result there were three different groups: patients presenting only infections (CHAPEL 1 = 61.2%), patients presenting autoimmunity and/or lymphocytic infiltration (CHAPEL 2-3 = 29.8%) and patients with enteropathy (CHAPEL 4 = 9.1%) (Figure 6B).

	CHAPEL 1	**CHAPEL 2-3**	**CHAPEL 4**	**p value**
N.	74	36	11	
Age, years (SD)	53.7 (14.1)	48.7 (15.4)	47.5 (11.4)	0.141
Gender – male, n (%)	31 (42.5)	17 (47.2)	6 (54.5)	0.717
BMI, kg/m^2 (SD)	25.9 (5.6)	25.5 (5.0)	22.9 (2.9)	0.224
Waist circumference, cm (SD)	94.5 (13.5)	96.2 (14.0)	99.8 (18.2)	0.210
SBP, mmHg (SD)	130 (15.4)	126 (14.7)	127 (9.4)	0.438
DBP, mmHg (SD)	78 (8.3)	75 (7.6)	76 (11.7)	0.307
Total cholesterol, mg/dL (SD)	204 (41.2)	178 (33.0)	200 (42.8)	**0.030**
LDL-cholesterol, mg/dL (SD)	129 (42.0)	109 (33.8)	125 (39.0)	0.126
HDL-cholesterol, mg/dL (SD)	56 (14.9)	49 (15.9)	51 (8.2)	0.181
Triglycerides, mg/dL (SD)	111 (54.8)	131 (100.9)	141 (81.6)	0.341
Glycemia, mg/dL (SD)	98 (17.6)	89 (14.7)	91 (16.8)	0.111
HbA1c, mmol/mol (SD)	38 (6.0)	33 (4.3)	35 (7.6)	**0.012**
Creatinine, mg/dL (SD)	0.8 (0.2)	0.8 (0.3)	0.8 (0.1)	0.947
eGFR, mL/min/1.73m^2 (SD)	91.1 (16.9)	95.7 (27.9)	99.9 (12.1)	0.362
Microalbuminuria, mg/dL (SD)	8.6 (9.4)	5.6 (6.6)	2.1 (2.1)	0.300
Serum sodium, mEq/L (SD)	139 (3.7)	139 (3.5)	141 (0.7)	0.542
CRP, mg/dL (SD)	4.0 (4.5)	3.4 (3.6)	4.5 (6.6)	0.817

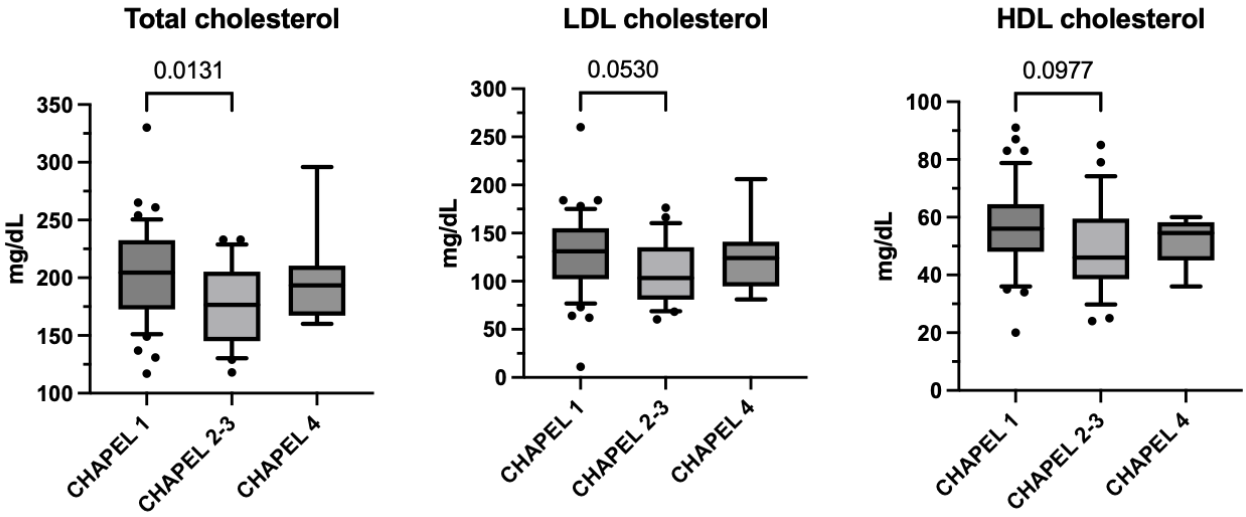
Table 6 ANOVA of general characteristics and humoral biochemical data between CHAPEL groups. Data are expressed as means ± standard deviation (SD). Data are expressed as means ± standard deviation (SD) or number of patients and percentage (%). BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low density lipoprotein; HDL, high density lipoprotein; CRP, C-reactive protein.

Patients present similar age and gender above groups (Table 11). Even BMI and blood pressure, both systolic and diastolic, were similar. Total cholesterol was significantly lower in patients with autoimmunity and/or lymphocytic infiltration (p=0.012) while LDL and HDL cholesterol, even if

seems to be lower, showed no significant difference above groups (Figure 7 and Table 11). In patients of group CHAPEL 2-3, glycemia and HbA1c were lower than other groups even if the percentage of patients taking glucocorticoid therapy was significantly higher (p=0.019). Even if the lipid-lowering treatment and diabetes therapy were not significantly different, when comparing lipid profile, glycemia and HbA1c, patients under those medications were excluded from the analysis (Table 12).

	CHAPEL 1	**CHAPEL 2-3**	**CHAPEL 4**	**p value**
N.	74	36	11	
Hypertension, n (%)	21 (31.3)	13 (39.4)	3 (27.3)	0.655
Hypertension therapy, n (%)	20 (29.9)	13 (39.4)	3 (27.3)	0.412
History of dyslipidemia, n. (%)	14 (20.6)	5 (16.1)	4 (36.4)	0.364
Hypercholesterolemia at first visit, n. (%)	37 (57.8)	14 (41.2)	5 (50.0)	0.290
Lipid-lowering therapy, n (%)	11 (16.4)	5 (15.6)	1 (10.0)	0.873
History of metabolic syndrome, n. (%)	13 (19.4)	10 (33.3)	2 (18.2)	0.297
Metabolic syndrome at first visit, n. (%)	18 (26.9)	14 (41.2)	3 (27.3)	0.326
Diabetes, n (%)	8 (12.5)	6 (18.2)	0 (0.0)	0.320
Diabetes therapy, n (%)	6 (9.2)	6 (18.2)	0 (0.0)	0.207
Glucocorticoids therapy, n (%)	8 (11.6)	11 (35.5)	2 (18.2)	**0.019**
Antinflammatory drugs, n (%)	2 (2.9)	0 (0.0)	1 (9.1)	0.276
Antiplatelet therapy, n (%)	5 (7.2)	1 (3.2)	3 (27.3)	**0.039**
Antibiotic prophylaxis, n (%)	6 (8.8)	5 (15.2)	1 (9.1)	0.618
Immunosuppressive treatment, n (%)	0 (0.0)	9 (27.3)	1 (9.1)	**<0.001**

Table 7 Comparison of drug therapy and risk factors between CHAPEL groups. Data are expressed as number of patients and percentage (%).



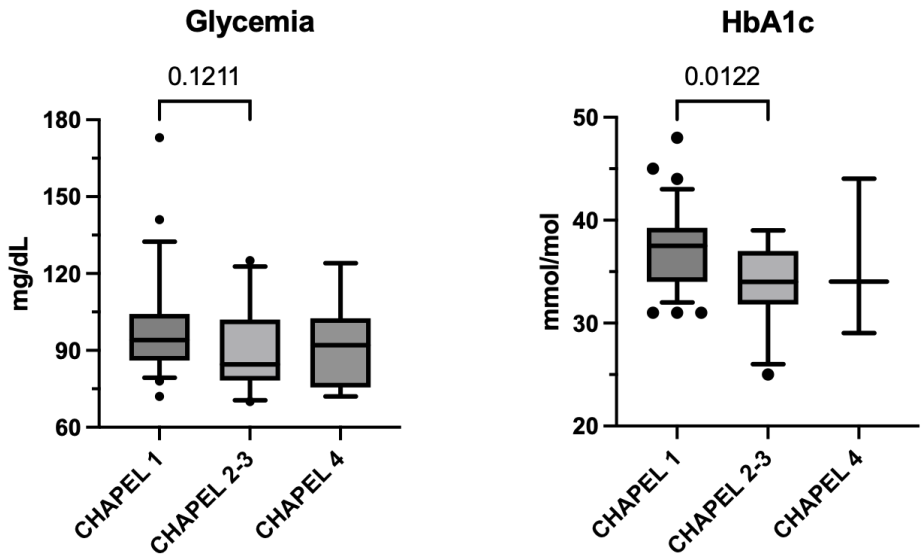


Figure 7 ANOVA of lipid profile, glycemia and HbA1c between CHAPEL groups. p value comparison between CHAPEL 1 and CHAPEL 2-3 was reported. Data are expressed as means ± standard deviation (SD).

	CHAPEL 1	**CHAPEL 2-3**	**CHAPEL 4**	**p value**
N.	74	36	11	
Previous CV events, n (%)	5 (6.8)	2 (5.6)	2 (18.2)	0.354
Smoke – active, n (%)	11 (15.7)	2 (6.1)	1 (9.1)	0.311
History of ACS, n (%)	1 (1.4)	0 (0.0)	1 (9.1)	0.130
History of stroke/TIA, n (%)	3 (4.3)	0 (0.0)	1 (9.1)	0.311
History of PAD, n (%)	1 (1.4)	0 (0.0)	0 (0.0)	0.725
History of TVP/EP, n (%)	2 (2.9)	2 (6.5)	0 (0.0)	0.540
History of carotid plaque, n (%)	4 (6.2)	0 (0.0)	0 (0.0)	0.278
History LVH, n. (%)	5 (7.2)	2 (6.3)	0 (0.0)	0.654
History of CKD, n. (%)	1 (1.4)	2 (6.5)	0 (0.0)	0.305
Age symptoms onset, years (SD)	34.2 (16.2)	27.4 (17.7)	28.6 (15.6)	0.217
Age diagnosis, years (SD)	42.7 (15.7)	39.8 (14.7)	34.3 (16.1)	0.286
Splenomegaly, n (%)	18 (26.9)	19 (55.9)	1 (9.1)	**0.003**
Splenectomy, n (%)	0 (0.0)	7 (20.6)	0 (0.0)	**<0.001**
Hepatomegaly, n (%)	4 (6.1)	2 (5.9)	0 (0.0)	0.705
Cancer, n (%)	17 (25.4)	8 (23.5)	0 (0.0)	0.170
GLILD, n (%)	1 (1.5)	19 (54.3)	0 (0.0)	**<0.001**
Bronchiectasis, n (%)	22 (32.4)	17 (50.0)	1 (9.1)	**0.034**
Autoimmunity, n (%)	15 (22.1)	17 (51.5)	4 (36.4)	**0.011**
ITP, n (%)	1 (1.5)	12 (35.3)	0 (0.0)	**<0.001**

Table 8 ANOVA comparison of previous cardiovascular events and disease related complications between CHAPEL groups. Data are expressed as means ± standard deviation (SD) or number of patients and percentage (%). ACS, acute coronary syndrome; TIA, transitory ischemic event; PAD, peripheral artery disease; VTE, venous thromboembolism; LVH, left ventricular hypertrophy; CKD; chronic kidney disease; CVID, common variable immunodeficiency; GLILD, Granulomatous Lymphocytic Interstitial Lung Disease; ITP, autoimmune thrombocytopenia.

Previous cardiovascular events were similar between groups, like history of ACS, TVP/EP and PAD; a slightly higher percentage in history of carotid plaque was found in patients presenting only infections (CHAPEL 1), possibly due to the higher lipid profile founded in those patients (Table 13). Diabetes and kidney function markers like creatinine and eGFR were similar above groups, the same for CKD. After blood metabolic evaluation, the calculated prevalence of metabolic syndrome markedly increased in all groups compared to the anamnestic information (from about 25% to about 33%). Disease related complications like GLILD ($p<0.001$), autoimmunity ($p=0.011$) and splenectomy ($p=0.003$) reflects the clinical classification used to make groups. In particular, ITP showed a very high prevalence in group CHAPEL 2-3 ($p<0.001$) (Table 13).

	CHAPEL 1	**CHAPEL 2-3**	**CHAPEL 4**	**p value**
N.	74	36	11	
Lymphocytes, % (SD)	32.5 (10.6)	32.4 (12.0)	31.1 (6.7)	0.941
CD3, % (SD)	74.9 (12.2)	79.3 (11.3)	78.9 (9.3)	0.220
CD4, % (SD)	42.8 (13.8)	41.3 (10.6)	45.8 (12.8)	0.640
CD8, % (SD)	33.4 (11.3)	39.5 (13.1)	31.4 (9.4)	0.052
CD4/CD8, % (SD)	1.4 (0.9)	1.3 (0.8)	1.8 (0.7)	0.334
CD3+CD8+CD57+, % (SD)	14.5 (11.7)	25.4 (13.4)	15.0 (11.2)	**0.001**
CD19, % (SD)	7.6 (6.1)	6.5 (5.3)	9.1 (8.9)	0.448
B naïve CD27-IgM+IgD+, % (SD)	68.5 (20.7)	77.6 (15.7)	65.9 (13.5)	0.107
B marginal CD27+IgM+IgD+, % (SD)	17.9 (16.1)	11.5 (11.7)	19.7 (13.2)	0.170
B switched memory CD27+IgM-IgD-, % (SD)	8.5 (8.2)	3.9 (4.7)	7.8 (4.8)	**0.024**
B transitional CD38++IgM++, % (SD)	0.9 (0.9)	2.8 (6.4)	1.9 (1.8)	0.063
B plasmablasts CD38++IgM-, % (SD)	0.3 (0.5)	0.5 (1.5)	0.2 (0.2)	0.568
B activated $CD21^{low}CD38^{low}$, % (SD)	6.8 (8.4)	8.9 (8.9)	5.3 (1.8)	0.428

Table 9 ANOVA comparison of immunological features between CHAPEL groups. Data are expressed as means ± standard deviation (SD).

Regarding immune cells analysis, patients belonging to group CHAPEL 2-3 showed significantly higher levels of CD3+CD8+CD57+ cells ($p=0.001$), known as large granular lymphocytes, often associated with autoimmunity. CD8+ cells showed slightly but not significant increase in the same group while number of total CD19+ cells was similar above groups (Table 14). Those patients presenting autoimmunity and/or lymphoproliferation showed significantly lower levels of IgG at diagnosis ($p=0.024$) (Table 15) and B switched memory CD27+IgM-IgD- ($p=0.024$) (Table 14).

	CHAPEL 1	CHAPEL 2-3	CHAPEL 4	p value
N.	74	36	11	
IgG diagnosis, mg/dL (SD)	382 (183.0)	266 (160.1)	366 (147.6)	**0.024**
IgA diagnosis, mg/dL (SD)	44 (44.4)	28 (38.0)	27 (25.6)	0.205
IgM diagnosis, mg/dL (SD)	80 (179.5)	47 (64.2)	31 (31.7)	0.469
IGRT, n (%)	58 (87.9)	32 (100.0)	11 (100.0)	0.060
IGRT initial dose, mg/kg/4week (SD)	324 (92.4)	320 (67.5)	374 (326.6)	0.734
IGRT steady state, mg/kg/4week (SD)	342 (91.1)	392 (105.8)	490 (413.9)	**0.023**
IgG steady state, mg/dL (SD)	760 (270.9)	823 (226.6)	813 (190.8)	0.473
IgA steady state, mg/dL (SD)	43 (61.3)	18 (25.5)	27 (26.9)	0.085
IgM steady state, mg/dL (SD)	67 (197.1)	56 (98.5)	46 (52.6)	0.917

Table 10 ANOVA comparison of immunoglobulin levels and replace therapy between CHAPEL groups. Data are expressed as means ± standard deviation (SD) or number of patients and percentage (%). IGRT, immunoglobulin replace therapy.

Vascular stiffness evaluation showed no significant difference above groups even if PP as AI seems to be higher in patients presenting only infections. Even AP shows a slightly increase in those patients suggesting that vascular stiffness could be more apparent (Table 16).

	CHAPEL 1	CHAPEL 2-3	CHAPEL 4	p value
N.	33	17	5	
Age, years (SD)	55.0 (13.5)	47.0 (14.6)	50.2 (10.2)	0.155
Gender – male, n (%)	9 (27.3)	8 (47.1)	2 (40.0)	0.365
BMI, kg/m^2 (SD)	26.8 (6.1)	23.9 (3.2)	23.2 (3.6)	0.149
Smoke – active, n (%)	3 (9.4)	1 (6.7)	0 (0)	0.775
LVH, n. (%)	3 (9.4)	0 (0)	0 (0)	0.370
History of metabolic syndrome, n. (%)	6 (19.4)	4 (28.6)	1 (20.0)	0.783
Diabetes, n (%)	4 (13.3)	2 (13.3)	0 (0)	0.685
Hypertension, n (%)	10 (31.3)	5 (33.3)	1 (20.0)	0.851
SP, mmHg (SD)	123 (16.4)	117 (14.6)	119 (11.1)	0.382
DP, mmHg (SD)	79 (9.6)	67 (8.6)	80 (17.7)	0.604
PP, mmHg (SD)	44 (9.7)	40 (13.1)	39 (10.4)	0.351
MAP, mmHg (SD)	99 (12.8)	94 (9.5)	97 (17.1)	0.410
HR, bpm (SD)	72 (12.5)	75 (17.6)	78 (16.5)	0.581
AP, mmHg (SD)	17 (7.4)	13 (9.0)	12 (4.5)	0.173
AIx, % (SD)	38 (13.0)	31 (14.1)	31 (7.9)	0.165
AIx 75, % (SD)	36 (13.6)	33 (15.4)	33 (10.2)	0.747
PWV, m/s (SD)	6.3 (1.3)	5.8 (0.7)	6.6 (1.5)	0.199
IMT DX, mm (SD)	0.6 (0.2)	0.6 (0.1)	0.7 (0.1)	0.413
IMT SIN, mm (SD)	0.7 (0.1)	0.6 (0.1)	0.8 (0.0)	0.103

Table 11 ANOVA comparison of vascular stiffness and atherosclerosis markers between CHAPEL groups. Data are expressed as means ± standard deviation (SD) or number of patients and percentage (%). BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure; HR, heart rate; AP, augmentation pressure; Aix, augmentation index; PWV, pulse wave velocity; IMT, intima-media tickness.

Gene expression analysis of LDLr and MR in untouched total CD19+ B cells isolated from peripheral blood was performed by ddPCR in 31 patients of CHAPEL 1, 13 patients of CHAPEL 2-3 and 7 patients in CHAPEL 4. MR and LDLr copies/UL normalized on GAPDH copies/uL did not show significant difference above groups even if in CHAPLE 2-3 gene expression of LDLr was higher (Figure 8).

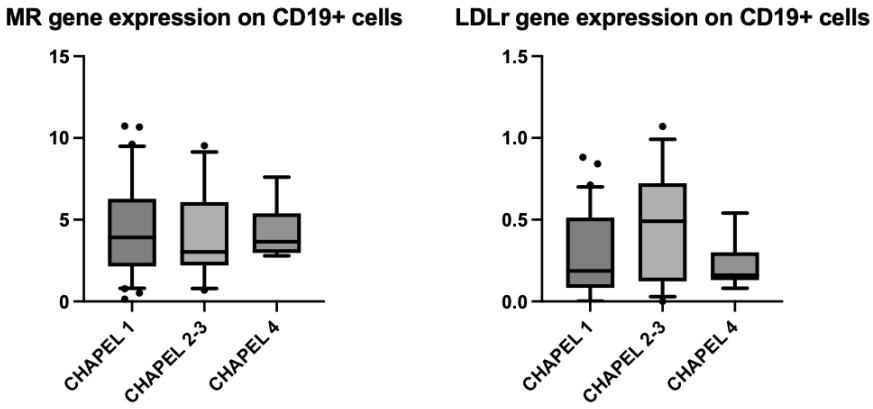


Figure 8 ANOVA comparison of LDLr and MR gene expression on CD19+ B cells between CHAPEL groups. Data has been normalized on GAPDH gene expression.

DISCUSSION

CVID is a rare disease, with less than 2000 patients living in Italy with a definite diagnosis. Infections-related mortality has been reducing over the last 20 years, due to optimization and personalization of immunoglobulin replacement strategies. Also due to the increase in life expectancy, cancer has now become the leading cause of death. For the same reason, atherosclerotic cardiovascular disease might become an increasing concern in patients now getting older and possibly being an at-risk population for atherosclerotic cardiovascular disease (ASCVD), mainly due to a chronic pro-inflammatory status. CVID patients commonly undergo a lifelong clinical, biochemical and instrumental screening strategy for early detection and management of infection and non-infection-related complications. These include regular lung function assessments, gastrointestinal endoscopies, chest high resolution CT scans and abdomen ultrasounds. To date, there are no data about the cardiovascular disease burden in cohorts of subjects presenting CVID and no specific cardiovascular disease assessment strategies are recommended. This is, to our knowledge, the first study that evaluates the cardiovascular risk and damage involving a large cohort of patients with common variable immunodeficiency. Interestingly, CVID patients might represent a highly suitable population to investigate the possible role of immunity, and particularly of B cells, in cardiovascular disease development. However, CVID is characterized not only by a depletion of B cell function and impairment in antibody response, leading to recurrent infections and to a consequently significant inflammatory burden, but also by B cell dysregulation and autoimmune phenomena (Patuzzo et al.). All these lead to a “variable” phenotype, as highlighted by the name of the disease itself. Cardiovascular diseases are well known to be driven by inflammatory status and B cells have been shown to be involved in the pathogenesis of atherosclerosis and vascular damage, even if mechanisms are not yet fully elucidated (Frostegård). B cells modulation has been described to significantly impact on cardiovascular disease progression. As an example, experimental depletion of B cells shows to attenuate plaque development and modulate T cell responses in mice (Pattarabanjird et al.) and to induce a significant reduction in the atherosclerosis process (Ait-Oufella et al.). In vivo study on patients presenting systemic lupus erythematosus showed that B cell depletion therapy improves the cardiovascular disease burden (Koulouri et al.). Emerging data are also demonstrating that different immunoglobulin classes can exert a significant impact on the cardiovascular disease development, e.g. by binding oxidized LDL (Sage et al.), modulating endothelial function and affecting insulin sensitivity (Napoli et al.). All these considered, patients

presenting CVID might represent an interesting *in vivo* model to investigate the role of B cells and the impact of immunoglobulins on cardiovascular disease development. Since data are lacking and based on physiopathological reasoning, in CVID patients we could have expected either an accelerated ASCVD, due to a chronic pro-inflammatory status, or a reduced cardiovascular disease burden, due to the lack of the pathogenic function of B cells in the atherosclerotic process.

To investigate these aspects, we designed a retrospective-prospective single-center study, that evaluated the 10-years prevalence of cardiovascular diseasein CVID patients by examining both risk factors and vascular damage development. Considering the rare frequency of the disease, estimated between 1 : 10 000 to 1 : 100 000, we enrolled around 7% of Italian affected subjects. In terms of immunologic characterization, as expected, enrolled patients showed a significant decrease in both IgG and IgA with a decrease also in CD19+ B cells and in particular of switched-memory B cells. Prevalence of hypertension was 34.2% (28% of male, 38% of female) while 13% of patients present diabetes. Those data are quite lower compare to data reported by "Progetto Cuore" for the Italian general population in the age range between 45 and 54 years, showing hypertension in 46% of male and 27% of female (age range 45-54 years) while 8-12% prevalence of diabetic subjects (data from "*Progetto Cuore"*). Also metabolic syndrome prevalence was in line with that described among Italian population (Miccoli et al.). Previous cardiovascular events occurred in 7.1% of our patients. This percentage seems to be lower if compared to what reported for European population (Townsend et al.). These data have to be considered in light of the young age of enrolled population (51.6 years), suggesting that those patients might be more suitable for evaluating risk factors and cardiovascular damage progression rather than prevalence of already occurred acute cardiovascular events. One possible explanation of the different prevalence in cardiovascular risk factors could be related to the clinical or immunological phenotype of these patients. For this reason, we decided to investigate whether features of clinical phenotype or immunological determinants may correlate with different cardiovascular risk profiles. As previously described, CVID patients can present different clinical phenotypes; the most common is characterized by recurrent infections (Infections only, CHAPEL 1) while others present a complicated phenotype, including autoimmune cytopenia (CHAPEL 2), lymphoproliferation (CHAPEL 3) and enteropathy (CHAPEL 4). Due to the chronic inflammatory status coupled with major dysregulation of immune system, we might have expected to find an overall worse cardiovascular risk profile in patients with complications. Moreover, complicated patients showed higher prevalence of glucocorticoids use, due to autoimmune manifestations, that is well known to affect glucose levels

in blood; on the other hand, the use of immunosuppressive treatment was also higher, with a potential overall impact on the function of different lymphocyte populations. Surprisingly, even if general characteristics (age, sex, BMI) were similar between patient with and without complicated phenotype, we found that total cholesterol, LDL cholesterol, HDL cholesterol, glycemia and HbA1c were significantly lower in patients presenting complications, also after excluding those under lipid-lowering treatment or diabetes therapy.

Subsequently, we focused on studying lymphocyte subpopulations distribution in these two subgroups of patients, finding significantly higher levels of CD3+CD8+CD57+ large granular T lymphocytes and lower levels of switched memory CD27+IgM-IgD- cells B cells in subjects presenting complications. Complicated CVID patients also presented a tendence towards higher percentage of activated $CD21^{low}CD38^{low}$ B cells and lower percentage of marginal zone CD27+IgM+IgD+ B cells. Expansion of large granular T lymphocytes is known to be associated with different autoimmune complications in immunocompetent subjects as well as in CVID patients (Barila' et al.), while reduction in switched memory B cells is related to a deeper impairment in IgG and IgA antibody production in CVID patients (Wehr et al.), that was actually shown in our cohort and that leads to higher requirements in terms of Ig replacement therapy. Expansion of $CD21^{low}CD38^{low}$ B cells have been reported in different immuno-rheumatologic diseases (e.g., rheumatoid arthritis) and associated with autoimmune manifestations in CVID patients, while reduction in marginal zone CD27+IgM+IgD+ B cells has been associated with higher infectious risk in CVID patients (Wehr et al.). Noteworthy, patients presenting autoimmune cytopenia and lymphoproliferation (CHAPEL 2 and 3) were found to be a particularly homogeneous subcohort in terms of metabolic and immunologic profile, while showing a higher frequency of genetic polymorphisms associated to CVID. This specific subgroup might thus offer the opportunity to further assess the impact of immunologic and genetic features on the cardiovascular disease development.

The key question is, at this point, whether these differences in the immunologic background may directly or indirectly explain the lower cholesterol and glucose levels found in the subgroup of complicated CVID patients, and if this may in turn influence the progression of ASCVD over time. At present, no direct relations between large granular T lymphocytes expansion and atherosclerotic cardiovascular disease have been described. The dysregulated function of $CD21^{low}CD38^{low}$ B cells, an antigen experienced population with potential APC function and an exhausted phenotype, has not been deeply investigated in the setting of ASCVD, while marginal zone CD27+IgM+IgD+ B cells have been suggested to play a potentially protective function (Shelby, Cells, 2021). The role of

switched memory B cells has not been directly investigated in ASCVD, but the role of IgG and IgA produced after class switch, as well as IgM produced before the switch, is still under investigation (Sage et al.). Interestingly, Napoli *et al.* suggested that immunoglobulins can directly stimulate in vitro nitric oxide (NO) production by endothelial cells and that a single intravenous administration of IgRT can exert a direct in vivo regulation on endothelial function, while improving insulin sensitivity in CVID patients (Napoli et al.). The majority of our patients receive immunoglobulin replacement therapy by intravenous or, more often, subcutaneous administration. Thus, a potential effect of IgRT on the endothelial function and on the insulin sensitivity should also be considered, both as an immediate effect after a single intravenous administration and as a long term effect due to regular intravenous and subcutaneous infusions.

Moving back to B lymphocytes, mechanisms through which B cells can recognize, remove and present lipids are not completely clear. It has been reported that B cells can recognize and remove lipids through different receptors, of which the most debated is LDLr. It has also been described that B lymphocytes purified from peripheral blood are able to internalize LDL with a four-fold increase in the expression of this receptor compared with non-stimulated T and NK cells (De Sanctis et al.). It is also clear that a defect in the expression of LDLr leads to an increase in circulating plasma LDL, leading to the oxidation process that contributes to the physiopathology of atherosclerosis (Jialal and Devaraj). Moreover, LDL removal through LDLr might be relevant not only for B cell metabolism, but also for LDL antigen presentation, which could participate in the inflammatory process observed in atherosclerosis. Another important receptor involved in the development of cardiovascular disease is mineralocorticoid receptor (MR). To date there are no data about gene expression of MR on B cells except for one study of *Armanini et al.* in 1988 (Armanini et al.), even if its pro-inflammatory effects are well known. MR in fact regulates macrophage activation to the proinflammatory M1 phenotype (Usher et al.). Moreover, MR activation promotes T lymphocyte differentiation to the pro-inflammatory Th1 and Th17 subsets while decreasing anti-inflammatory T regulatory lymphocytes (Amador et al.). Based on these previous findings, we decided to investigate B lymphocytes gene expression of those two receptors in order to explore possible mechanisms of direct influence of B cells gene expression and function on the regulation of lipid metabolism. Preliminary results of gene expression analysis in a limited subgroup of subjects confirmed the expression of both MR and LDLr in B cells isolated from peripheral blood of CVID patients. In the subgroup of patients who have already undergone gene expression analysis, no difference of MR expression was found in relation to the clinical phenotype.

A difference was instead found, even if not statistically significant, when comparing LDLr expression in patients with different clinical phenotypes. In fact, patients with complicated phenotype (who presented lower levels of lipid profile) showed higher levels of LDLr gene expression analysis when compared to the "infection only" subgroup. This finding deserves further investigation but, on the basis of the difference in distribution of B cell subtypes between patient with distinct clinical phenotypes, may at least suggests a different expression of LDLr in B cell subsets, with potential implication on the regulation of lipid profile. Based on this preliminary analysis performed in a limited number of patients, it could be thus interesting to explore protein expression on cell surface after B cell subpopulations sorting, as well as on large granular lymphocytes.

Finally, we decided to take an *in vivo* baseline picture of vascular damage in CVID patients by evaluating pulse wave velocity (PWV) and pulse wave analysis (PWA). Pulse wave velocity is commonly used as an effective noninvasive indicator for assessing arterial stiffness, in fact, an increase in PWV is associated with large-artery stiffness, a marker of vascular damage and predicts the risk of CV event (Sequí-Domínguez et al.). PWA is a technique that allows the accurate recording of peripheral pressure waveforms and generation of the corresponding central waveform, from which the augmentation index and central pressure can be derived (O'Rourke and Jiang). Carotid ultrasound examinations were performed to establish the presence of carotid plaques and to measure intima media thickness (IMT). Vascular damage evaluation was performed using non-invasive pulse wave analysis monitoring system SphygmoCor Xcel (AtCor Medical, Australia) in 55 CVID patients. In order to avoid any possible confounders, according to literature, PWV comparison was performed after adjustment for HR and MAP while AP and AIx comparisons were performed after adjustment for HR and body height (Van Trijp et al.). Overall, subjects presenting a complicated clinical phenotype showed lower values of all stiffness markers with a significantly lower pulse pressure and augmentation pressure compared to subjects with only infections. The guidelines of the European Society of Cardiology on cardiovascular disease define presence of plaque with an IMT $\geq$ 1.5mm (Visseren et al.), mean IMT of our patients was 0.5 – 0.7 mm. This data, limited by the low number of tested subjects, suggest that patients presenting clinical complications may present vascular stiffness less than similar age, sex and risk factors patients with immunodeficiency related complications.

Taken together, these data suggest that, in a cohort of patients with primary B cell defects, those with major clinical and immunological signatures of immune dysregulation may present a better cardiovascular risk profile compared to subjects with "only infections" phenotype. The better

cardiovascular profile is defined by a lower lipid profile, a lower glucose levels and a tendency of lower vascular stiffness. Moreover, the differences in lymphocytes subsets distribution, immunoglobulin levels and Ig replacement therapy between these groups, raise the hypothesis that these factors may directly or indirectly impact on atherosclerotic cardiovascular disease development.

This study presents some limitations. The first one is the relatively low number of enrolled subjects, of which only a subgroup underwent vascular evaluation by *in vivo* studying stiffness and IMT and gene expression analysis. Considering the prevalence of CVID in the general population, however, these results may at least drive some preliminary speculations.

Another limitation is the absence of a control group of immunocompetent subjects with matched age and sex, to compare cardiovascular and immunologic features. Moreover, to better explore if the type of immunodeficiency may exert an active role in regulating cardiovascular disease development, other control groups could be considered, e.g. subjects with other primary immunodeficiencies or secondary antibody deficiencies.

Another important aspect is linked to the mean age of our population, that may limit the possibility to evaluate incidence or cardiovascular events or direct *in vivo* signs of vascular damage. However, after this baseline picture, such a cohort will undergo long term clinical and instrumental follow-up, hopefully revealing the relationship between immunologic and cardiovascular features over time. Finally, to better investigate the possible role of immunoglobulins in the cardiovascular disease development and their long-term effect on the endothelial function, the ongoing prospective study is recruiting new patients at the time of CVID diagnosis and before treatment initiation; this will allow comparison between pre-treatment and in-treatment parameters, hopefully disclosing the long-term impact of IgRT on metabolic profile and ASCVD.

To conclude, prevalence of cardiovascular disease in CVID patients is apparently in line with that observed in general population, but a subgroup of patients with specific clinical and immunologic features and receiving higher dosage of IgRT may present a more favorable cardiovascular risk profile. Considering the relatively young age of the cohort and that CVID onset commonly occurs at young adult age, long term follow-up will unravel whether this may lead to a lower degree of vascular damage and to a lower incidence of acute cardiovascular events. Understanding if our preliminary data will translate into different clinical ASCVD development will help us to clarify the role of B lymphocytes and immunoglobulins in the pathogenesis of cardiovascular disease. Those information may in turn have a high impact value not only in defining

specific cardiovascular follow-up strategies for CVID patients, but also in designing personalized prevention and treatment strategies for high-cardiovascular-risk immunocompetent subjects, based on immunologic profiling and taking advantage from an increasing range of available B cells targeting drugs.

SUPPLEMENTARY

Table S1.

Gene	Sequences
hLDLr	For: 5’- TCTATGGAAGAACTGGCGGC - 3’
	Rev: 5’- ACCATCTGTCTCGAGGGGTA - 3’
MR	For: 5’- GATGGTAACTAAGTGTCCCAACAA - 3’
	Rev: 5’- TTCCAGCAGGTCGCTCACCAGG- 3’
GAPDH	For: 5’- AGCCACATCGCTCAGACAC - 3’
	Rev: 5’- GCCCAATACGACCAAATCC - 3’

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