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OCULAR AND GENETIC CHARACTERISTICS OF PATIENTS SURVIVING LONG-LASTING TYPE 1 DIABETES WITHOUT VASCULAR COMPLICATIONS

DoS: Prof. Giuseppe Querques

Second Supervisor: Dr. Michael Ip

Tesi di DOTTORATO di RICERCA di Enrico Borrelli

matr. 015686

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Il sottoscritto	Enrico Borrelli
Matricola/*registration number*	015686
nato a/ *born at*	Bari
il/*on*	05/04/1988

autore della tesi di Dottorato di ricerca dal titolo / *author of the PhD Thesis entitled*

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- Whole-exome sequencing (WES) analyses were performed in collaboration with Center for Omics Sciences (COSR), IRCCS Ospedale San Raffaele, Milan Italy; and Drs. Silvia Galbiati and Gianpaolo Zerbini, Complications of Diabetes Unit, Diabetes Research Institute, IRCCS Ospedale San Raffaele, Milan, Italy.
- Serum C-peptide analysis was performed in collaboration with Drs. Silvia Galbiati and Gianpaolo Zerbini, Complications of Diabetes Unit, Diabetes Research Institute, IRCCS Ospedale San Raffaele, Milan, Italy.

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ABSTRACT

Background and aims: Recent evidences suggest that a group of patients with either type 1 or type 2 diabetes may be spared by microvascular complications, even after a long-lasting disease. The aim of this study was to investigate the clinical and genetic characteristics of patients with long-lasting (i.e. > 30 years) type 1 diabetes who are not affected by systemic microvascular complications. The characterization of this very rare niche of patients may reveal new insights into the diabetes-associated microvascular complications' pathogenesis.

Material and methods: In this case-control study, Caucasian patients with type 1 diabetes for at least 30 years and without evidence or history of systemic microvascular complications (i.e. diabetic retinopathy [DR] or diabetic nephropathy) were prospectively enrolled. Patients underwent a complete ophthalmological assessment, including structural optical coherence tomography (OCT) and OCT angiography (OCTA). Furthermore, blood samples were obtained in all subjects. OCT and OCTA images were analyzed with previous validated algorithms in order to quantify inner and outer retinal thicknesses, as well as retinal and choroidal perfusion. For the latter analyses, a control group of 30 healthy controls was included for comparisons. Blood samples were analyzed with whole-exome sequencing (WES) in order to determine whether in our study cohort there were variants in well-studied genes that were previously suggested to be associated (i.e. either protective or causative) with diabetes-associated microvascular complications, as well as to investigate presence of causative or protective variants.

Results: Twenty-seven subjects (12 females, 15 males) with long-lasting type 1 diabetes and without microvascular complications were included. Mean±SD age was 51.4±9.5 years. Mean±SD duration of diabetes was 37.7±6.5 years. We selected for rare missense variants that were either protective or causative for DR. No differences in OCT and OCTA metrics were detected between patients and healthy controls.

Conclusions: This study identified genes that seem to be associated with a lower vs. greater risk of developing DR. The identification of these genes might grant strategic and personalized therapeutic plans in patients with diabetes. Finally, our patients did not show retinal structural and/or vascular changes, the latter finding suggesting that these patients may be protected by clinical and subclinical alterations.

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1 ACRONYMS AND ABBREVIATIONS

AC – allele count

ANCOVA – analysis of covariance

BCVA – best-corrected visual acuity

CC – choriocapillaris

DCP – deep retinal capillary plexus

DNA – deoxyribonucleic acid

DR – diabetic retinopathy

FAZ – foveal avascular zone

FD% – percentage of flow deficits

GMAF – global minor allele frequency

LogMAR – Logarithm of the Minimum Angle of Resolution

OCT - optical coherence tomography

OCTA - optical coherence tomography angiography

PD – perfusion density

PDR – proliferative diabetic retinopathy

SCP – superficial retinal capillary plexus

SD – standard deviation

SS – swept source

VLD – vessel length density

WES – whole-exome sequencing

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3 INTRODUCTION

The prevalence of diabetes has dramatically risen worldwide and eye complications secondary to diabetes represent a global health issue which may affect patients' vision (Bandello *et al*, 2017; Querques, 2019). Although available therapies for diabetic retinopathy (DR)-associated complications may ameliorate the visual outcome of diabetic patients,(American Diabetes Association, 2014) this condition is still considered as a leading cause of blindness.

Recent evidences suggest that a group of subjects with either type 1 or type 2 diabetes may be free of microvascular complications. In details, the Medalist study[3] showed that a subgroup of individuals with at least 50 years of type 1 diabetes were not affected by systemic vascular complications. Similarly, the Riace study (https://clinicaltrials.gov/ct2/show/NCT00715481 and http://www.siditalia.it/ricerca/centro-studi-e-ricerche/72-riace) confirmed that also a number of subjects with a long history of type 2 diabetes may be free of systemic microvascular complications. The latter findings may suggest that some clinical and/or genetic factors may protect diabetic patients from developing microvascular complications.

Several studies have employed structural optical coherence tomography (OCT) and OCT angiography (OCTA) imaging modalities to investigate the neural changes and retinal perfusion, respectively, in patients with diabetes.

Although DR is mainly considered as a vascular disease, there are many factors that were suggested to be involved in the pathogenesis of this disease. In detail, diabetes is known to have detrimental effects on the survival and function of cells in the inner retina (i.e. ganglion cells and amacrine cells) (Carpineto *et al*, 2016). Importantly, recent evidences have suggested that photoreceptors may also be characterized by early alterations in diabetes (Borrelli *et al*, 2019b). These neural changes have been suggested to occur both independently and secondarily to clinically observed retinal vasculopathies (Tombolini *et al*, 2022). More importantly, patients without DR were characterized by a thinner the inner retina, as demonstrated in OCT studies (Carpineto *et al*, 2016).

DR has been historically considered as a vascular disease as diabetes may result in damage of the retinal capillaries (capillary dropout) (Bresnick *et al*, 1975). Recently, OCTA has been broadly employed to provide a qualitative assessment and quantification of the macular perfusion in patients with diabetes. In detail, OCTA reports showed that DR eyes are featured by a larger foveal avascular zone (FAZ) and reduced macular perfusion at both the superficial retinal capillary plexus (SCP) and deep retinal capillary plexus (DCP) levels (Borrelli *et al*, 2020a). Importantly, OCTA evidences did also suggest that DR may be associated with a choroidal impairment as the choriocapillaris (CC) perfusion was also showed to be lower in patients with non-proliferative DR.[5] Finally, OCTA analysis displayed that diabetic patients without DR are featured by an impairment of the retinal perfusion (Sacconi *et al*, 2020a; Carnevali *et al*, 2017).

4 AIM OF THE WORK

What is lacking is information concerning the clinical and genetic characteristics of individuals with long-lasting (i.e. > 30 years) type 1 diabetes who are not affected by systemic microvascular complications. The identification and characterization of this very rare niche of patients may indeed reveal new insights into the diabetes-associated microvascular complications' pathogenesis.

5 RESULTS

Twenty-seven subjects (12 females, 15 males) with long-lasting type 1 diabetes and without any history and/or evidence of systemic microvascular complications were included. Mean±SD age was 51.4±9.5 years (median=51.5 years; range 34-68 years). Mean±SD duration of diabetes was 37.7±6.5 years (median=37 years; range 30-52 years). The best-corrected visual acuity (BCVA) was 0.0±0.0 LogMAR (Snellen equivalent ~20/20).

5.1 Whole-exome sequencing analysis in subects with long-lasting type 1 diabetes and no microvascular complications: comparison with literature (first step)

We sought to determine whether in our study cohort there were variants in well-studied genes that previous studies had suggested to be associated (i.e. either protective or causative) with diabetes-associated microvascular complications. List of candidate genes are reported in Table 1.

Table 1. List of candidate genes based on previous reports.

Gene	Chromosome
NME-3	16
FASTK	7
ABCA7	19
ABHD17A	19
ANO2	12
BPIFB6	20
C15orf32	15
CCDC105	19
CDKL1	14
CEP192	18
COL6A5	3
CRIPAK	4
DNHD1	11
GPATCH1	19
HMCN1	1
KIF24	9
LRBA	4
LRP8	1
MSH2	2
NAT1	8
PHF21A	11
PKHD1L1	8

SLC6A13	12
SLURP1	8
TTC22	1
UPK3A	22
VPS13B	8
ZDHHC11B	5
ZDHHC11	5
ZNF600	19
AKR1B1	7
AGER	6
VEGFA	6
NOS3	7
ACE	17
EPO	7
CACNB2	10
CAMK4	5
FMN1	15
GRB2	17
NVL	1
STT3B	3
PALM2AKAP2	9
Intergenic locus in between AKT3 and ZNF238	1
ZNF395	8
COL18A1	21

AKR1B1/ALR2	7
ITGA2 (α2β1 integrin)	5
AGTR1	3
ADRB3	8
AGT	1
APOE	19
FGF2	4
SLC2A1	1
HLA	6
SDH (sorbitol dehydrogenase)	15
ICAM1	19
MTHFR	1
NPY	6
PAI-1	7
PON1	7
PPARG	3
VDR	12
EDN1 (endothelin-1)	6
ROCK2	2
CPVL/CHN2	7
FRMD3	9
CARS	11
IRS2	13
SOD2	1

MnSOD	6
CA (Carbonic anhydrase)	8
PEDF	17
HTRA1/ARMS2	10
CFH	1
PSMD9	5

Our analysis revealed that:

- One candidate gene, NAT1, had the variant *rs5030839* in only 2 diabetes patients without microvascular complications, while no patient with PDR had this variant. This variant was suggested to be causative of DR in a multiracial cohort of subjects with type 2 diabetes (Ung *et al*, 2017). Our results seem to suggest that this specific variant would be not associated with DR in our study cohort of Caucasian individuals with type 1 diabetes.
- One candidate gene, VEGFA, had the variant *rs25648* in 5 out of 27 (18.5%) patients without microvascular complications, while all patients with DR (3/3, 100%) displayed this specific variant. This variant was suggested to be causative of DR in a systematic review and meta-analysis on subjects with type 2 diabetes (Liang *et al*, 2013). This seems to suggest that this specific variant of VEGFA would be associated with a greater risk to develop DR in our study cohort of Caucasian patients with type 1 diabetes.
- One candidate gene, ACE2, had the variant *rs4343* in 15 individuals without microvascular complications (heterozygous and homozygous variants in 9 and 6 patients, respectively), while this variant was present in heterozigosis in 3/3 patients with DR. This variant was suggested to be causative of DR in a Chinese cohort of type 2 diabetes subjects (Liang *et al*, 2013). These findings may suggest that this variant is not associated with a greater risk of developing DR in our study cohort of Caucasian subjects with type 1 diabetes. However, this variant is characterized by a global minor allele frequency (GMAF) of 0.35683 and is thus common in the general population.
- One candidate gene, SOD2, had the variant *rs4880* in in 15 out of 27 (55.5%) patients without microvascular complications, while only one patient with DR (1/3, 33.3%) displayed this specific variant. Kuo and colleagues(Kuo *et al*, 2014) suggested that the rs5370 variant of EDN1 may be protective for the development of DR in a Chinese cohort of type 2 diabetes individuals. Our findings may suggest that this variant may be protective for DR in our study cohort of Caucasian patients with type 1 diabetes.
- One candidate gene, EDN1, had the variant *rs5370* in in 17 out of 27 (63.0%) patients without microvascular complications, while only one patient with DR (1/3, 33.3%) displayed this specific variant. This variant was suggested to be protective for the development of DR in a Chinese cohort of subjects with type 2 diabetes (Yang *et al*, 2020). Our findings may suggest that this variant may be protective for the DR developing in our study cohort of Caucasian patients

with type 1 diabetes.

5.2 Whole-exome sequencing analysis considering both subects with long-lasting type 1 diabetes and no microvascular complications and patients with PDR to determine plausible causative variants (second step)

As stated above, a control group of three patients (two females) with a diagnosis of proliferative diabetic retinopathy after a history of type 1 diabetes lasting less than 20 years was also included in the whole-exome sequencing (WES) analysis for comparisons. Mean±SD age was 34.7±13.3 years.

For this analysis, as stated above, we included only variants that were characterized by a minor allele frequency less than 0.1%. Moreover, we included only those variants that were likely to alter protein function (e.g. missense or loss-of-function mutations). Finally, variants were further included whether they were present in at least 2 out of three subjects in heterozygosis or homozygosis.

In our cohort of patients with PDR after a short duration of type 1 diabetes, we identified 9 candidate genes on the basis of this analysis:

- UQCRC2 (Ubiquinol-Cytochrome C Reductase Core Protein 2) encodes for a protein which is located in the mitochondrion as a part of the ubiquinol-cytochrome c reductase complex which is an element of the mitochondrial respiratory chain.
- NEFH (Neurofilament Heavy Chain) encodes for the heavy neurofilament protein. The latter protein is an element of the axoskeleton and is involved in maintaining the neuronal caliber.
- THOC5 (THO Complex 5) encodes for a protein which is part of the TREX complex, the latter involved in the coupling process of several processes

including mRNA transcription, and processing and nuclear export

- ASCC2 (Activating Signal Cointegrator 1 Complex Subunit 2) encodes for a protein which has a main role in DNA damage.
- CXCL16/ZMYND15 (Zinc Finger MYND-Type Containing 15) encodes for a MYND-containing zinc-binding protein. Studies on mice have revealed a similar gene which acts as a testis-specific transcriptional repressor.
- NEB (Nebulin) encodes for a protein named nebulin, which is a main component of the cytoskeletal matrix and has a main role in the sarcomeres of skeletal muscle.
- CCDC60 (two variants in cis) (Coiled-Coil Domain Containing 60).
- ZNF14 (Zinc Finger Protein 14).
- MTTP (Microsomal Triglyceride Transfer Protein) encodes for a protein which is part of a transfer proteinplaying a major role in lipoprotein assembly.

As specified above, only variants likely to alter protein function were selected.

5.3 Whole-exome sequencing analysis considering both subects with long-lasting type 1 diabetes and no microvascular complications and patients with PDR to determine plausible protective variants (third step)

In our cohort of subjects with long-lasting type 1 diabetes and no microvascular disturbances, we identified candidate genes with variants likely to alter protein function on the basis of their carrying alleles in as many subjects without DR as possible (at least 34 out of 54 alleles – allele count [AC] of at least 34) and none in the 3 patients with PDR:

- CLIP1 had missense *rs1129167* variant in 37 out of 54 alleles of the analyzed population. CLIP1 (CAP-Gly Domain Containing Linker Protein 1) encodes for a protein which stabilizes the growing, distal ends of microtubules. The latter protein is involved in different pathways including cell cycle.
- TRMT44 had missense *rs1880024* variant in 37 out of 54 alleles of the analyzed population. The protein encoded by TRMT44 is a putative tRNA methyltransferase found in the cytoplasm.
- RB1CC1 had missense rs17337252 variant in 34 out of 54 alleles of the analyzed population. The protein encoded by RB1CC1 is involved in coordinating cell growth and proliferation, as well as apoptosis and autophagy.

5.4 C-peptide

No significant differences in C-peptide levels were detected among groups (Figure 1).

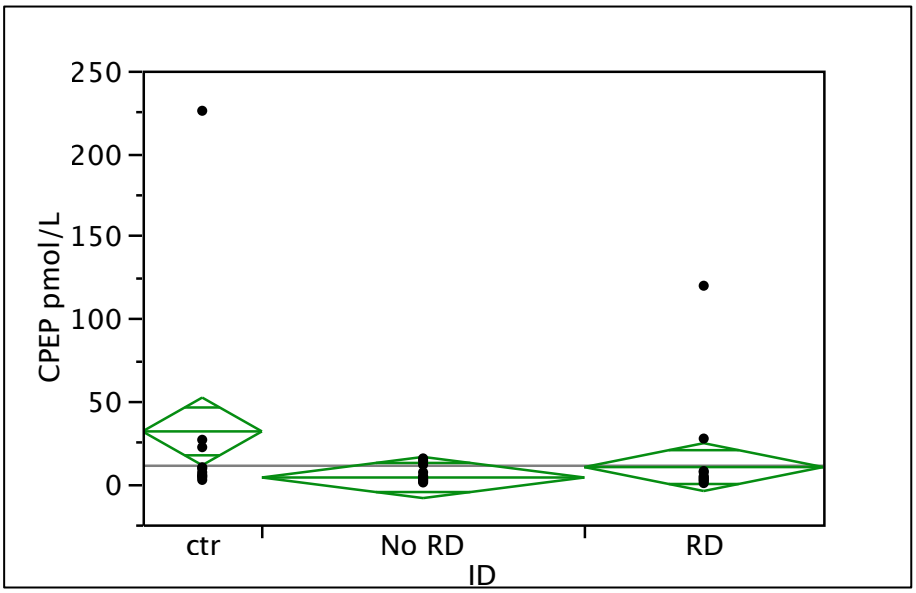

Figure 1. Diamond plots showing C-peptide levels in different groups*. The green diamonds illustrate the mean values and the 95% confidence interval.*

5.5 Structural OCT analysis

As stated above, a control group of 30 healthy controls 14 females, 16 males~~)~~ was included for OCT and OCTA comparisons. Mean±SD age was 53.8±10.4 years.

The foveal inner retinal thickness was 75.4±3.3 µm in patients with diabetes and 76.1±3.1 µm in control eyes (p=0.563). Similarly, no differences in thickness were found in the inner retina within the parafoveal area (141.2±4.9 µm and 142.7±2.8 µm, in diabetes and control groups, respectively, p=1.0).

5.6 OCTA analysis

At the SCP level, the parafoveal PD and VLD were similar in diabetic patients (35.1±1.8 % and 6.2±0.3 %) and healthy subjects (35.4±1.0 % and 6.4±0.3 %, p=1.0 and p=0.645). Similarly, both the DCP PD (32.6±2.3 % and 32.8±2.0 %, in diabetic and control subjects, respectively) and VLD (6.3±0.4 % and 6.4±0.2 %, in diabetes and control groups, respectively) did not differ between groups (p=1.0 and p=1.0, respectively). The CC FD% was similar between groups within the foveal (17.1±7.1 % in diabetic individuals vs. 16.7±7.9 % in controls, p=0.344) and parafoveal (15.2±5.9 % in diabetic patients vs. 14.9±6.4 % in controls, p=0.520) regions.

6 DISCUSSION

In this study we assessed the clinical and genetic characteristics of individuals with long-lasting (i.e. > 30 years) type 1 diabetes who are not affected by systemic microvascular complications. In order to characterize the genetic profile of these patients, we performed a WES analysis which also included an extreme phenotype of three patients complicated by PDR after a short duration of type 1 diabetes. In our analysis, we investigated a list of candidate genes that were either protective or associated with DR, as based on previous reports (Shtir *et al*, 2016; Ung *et al*, 2017; Bhatwadekar *et al*, 2021; Kuo *et al*, 2014). Also, we selected for rare missense variants that were either protective or causative for DR. Finally, our results showed that this very rare group of patients with long-lasting diabetes did not display subclinical structural and vascular modifications of the retina, as assessed using structural OCT and OCTA. Importantly, c-peptide levels did not differ among groups, which suggests that protection from DR is not secondary to basal levels of insulin.

As compared with whole-genome sequencing, whole-exome sequencing is a cost-effective methodology which allows to identify genetic variants. In details, WES focuses on protein-coding regions (exons) which contain the majority of mutations leading to disease-related traits, although they cover about 1% of the entire genome. Previous studies employing WES have investigated the genetic profile of diabetic subjects with and without DR. While the use of WES to assess patients with diabetes is still relatively limited, these previous reports demonstrated the potential of this methodology to further expand our insight into the diabetes-related microvascular complications.

In a previous study, Shtir et al(Shtir *et al*, 2016) analyzed 43 patients without DR after at least 10 years of disease who were compared with 64 patients with DR developed within 10 years after the diagnosis of diabetes. Of note, either group was from a Saudi Arabian cohort of type 1 or 2 diabetic patients. In the latter study, the authors showed that two genes (NME3 and FASTK) seem to be protective for developing of retinal vascular disease. In contrast, in our study cohort of Caucasian individuals with type 1 diabetes, these two genes seem to be not protective for DR.

This discrepancy might be secondary to different ethnic cohorts that may have contributed in the identification of different genes.

In a successive study, Ung et al(Ung *et al*, 2017) compared 43 patients who developed PDR with 13 individuals without clinical evidence of DR after 10 or more years of diabetes. Included subjects were from a multiracial cohort of type 2 diabetes individuals. The latter study investigated the presence of variants altering protein function and with a minor allele frequency lower than 0.1%. The authors identified 44 genes whose variants may be related with the DR developing. Among these, we did find the variant rs5030839 of NAT1 in only 2 diabetes patients without microvascular complications, while no patient with PDR had this variant. This seems to suggest that this specific variant would be not associated with DR in our Caucasian study cohort of type 1 diabetes patients.

Bhatwadekar and colleagues(Bhatwadekar *et al*, 2021) have recently provided a review of studies assessing genetic contribution to DR that have displayed putative candidate genes. Among the candidate genes to be associated with an increased risk of DR occurrence that were diaplayed in the latter review,(Bhatwadekar *et al*, 2021) we did find the variant *rs25648* of VEGFA(Yang *et al*, 2020) in 5 out of 27 (18.5%) patients without microvascular complications, while all patients with DR (3/3, 100%) displayed this specific variant. Therefore, our data seems to confirm that this variant of VEGFA may be associated with a greater risk to develop retinal vascular complications.

The rs4343 ACE variant was also reported to be related to DR.(Liang *et al*, 2013) In details, a case-control study analyzed 63 type 2 diabetes subjects without DR, 82 type 2 diabetic individuals with DR, and 90 age- and gender-matched healthy subjects. Results of the latter study showed that rs4343 ACE variant is associated with DR in Chinese subects with type 2 diabetes. In our study cohort of Caucasian patients with type 1 diabetes, we found that the variant *rs4343* of ACE2 characterized 15 individuals without microvascular complications (heterozygous or homozygous variants in 9 and 6 patients, respectively), while this variant was present in heterozigosis in 3/3 patients with DR. These findings may suggest that

this variant is not associated with a greater risk of developing DR. Moreover, this variant is characterized by global minor allele frequency (GMAF) quite common in the general population (0.35683). This discrepancy might be secondary to different ethnic cohorts and different type of diabetes.

In a review by Kuo et al,(Kuo *et al*, 2014) several gene variants were suggested to be associated with a either lower or greater risk of DR development. In the latter review, the rs4880 variant of SOD2 was suggested to be protective for the development of DR. In our study cohort, this variant was displayed in 15 out of 27 (55.5%) patients without microvascular complications, while only one patient with DR (1/3, 33.3%) displayed this specific variant. Therefore, our results seem to suggest that this variant may be protective for the development of DR. Similarly, Kuo and colleagues(Kuo *et al*, 2014) suggested that the rs5370 variant of EDN1 may be protective for the development of DR in a Chinese cohort of patients with type 2 diabetes. Accordingly, in our study cohort, this variant was found in 17 out of 27 (63.0%) patients without microvascular complications, while only one patient with DR (1/3, 33.3%) displayed this specific variant. These findings seem thus to further confirm that this variant may be protective for the development of DR, even in Caucasian subjects with type 1 diabetes.

In our cohort of patients, we identified 9 genes that seem to be causative of DR, on the basis of their carrying null het alleles in at least two PDR individuals and none in the group of patients without DR. In these patients, variants were filtered to alter protein function and with a minor allele frequency lower than 0.1%. Among the 9 genes whose mutations seem to be associated with a greater risk of DR development, ASCC2 (Activating Signal Cointegrator 1 Complex Subunit 2) was previously demonstrated to be associated with a greater risk of developing type 1 or 2 diabetes (Kaur *et al*, 2021). ASCC2 is involved in ubiquitin binding activity which might be responsible for commonly regulating β-cell function in human islets and contributing to both types of diabetes (Kaur *et al*, 2021). Therefore, our results might suggest that mutations in ASCC2 that alter protein function might affect residual beta cell function this conferring higher risk of retinopathy. Similarly, we did show that mutations in MTTP (microsomal triglyceride transfer protein) may be associated with a higher risk of developing DR. The latter gene encodes for a

protein playing a pivotal role in lipoprotein assembly and secretion and mutation of this gene promoter was demonstrated to be associated to a blunted insulin responsiveness (Au *et al*, 2008). Therefore, our results might suggest that mutations in MTTP may affect insulin responsiveness and thus increase risk of retinopathy.

In our cohort of patients, we identified 3 genes that seem to be protective for DR, on the basis of their carrying alleles in as many subjects without DR as possible (at least 34 out of 54 alleles – allele count of at least 34) and none in the 3 patients with PDR. Among the 3 genes whose mutations seem to be associated with a lower risk of DR development, RB1CC1 encodes for a protein that is involved apoptosis and autophagy. Emerging evidences have suggested that oxidative stress-related autophagy and apoptosis of cells in the retina may be comprised in the pathogenesis of DR and enhanced autophagy and apoptosis may accelerate DR progression (Chang & Chuang, 2010; Cai *et al*, 2017; Navarro-Yepes *et al*, 2014; Dimitrova *et al*, 2017). Therefore, our results might suggest that mutations in RB1CC1 that alter protein function might affect these processes and protect from DR development.

Several previous OCTA studies indicated that diabetic subjects with no DR signs are featured by an early macular hypoperfusion (Borrelli *et al*, 2021d; Tombolini *et al*, 2022). In details, the size of the FAZ was showed to be larger in those individuals with diabetes. Noteworthy, the increased size was showed to be significant even in patients without sings of DR (Vujosevic *et al*, 2019). Accordingly, a greater FAZ size was a showed in individuals with type 2 diabetes with no clinically detectable DR (De Carlo *et al*, 2015). Moreover, diabetic subjects without clinical signs of DR are also featured by a retinal neuronal loss (Carpineto *et al*, 2016; De Benedetto *et al*, 2014).

Several studies have used OCTA metrics to quantitatively show a reduced macular perfusion in diabetic subjects (Borrelli *et al*, 2021d, 2021a). OCTA analysis demonstrated that diabetic individuals with no evidence of DR are featured by a lower retinal perfusion in comparison with healthy normal controls.(Carnevali *et al*, 2017; Sacconi *et al*, 2020b)

In the present study, structural OCT and OCTA analyses was not able to demonstrate changes in diabetic patients, as compared with controls. These results may suggest that these patients are also spared by subclinical retinal changes.

Our study does have a number of limitations that include its sample size and cross-sectional nature. We may have failed to find significant associations because of a small simple size. Future large longitudinal studies may shed further light on the genetic profile of diabetic patients without microvascular complications although a long-lasting diabetes.

In conclusion, this study investigated a very rare group of Caucasian patients with long-lasting (i.e. > 30 years) type 1 diabetes who are not affected by systemic microvascular complications. This study identified genes that seem to be associated with a lower vs. greater risk of developing DR. The identification of these genes might grant strategic and personalized therapeutic plans in patients with diabetes. Future larger studies employing Sanger sequencing may confirm whether variants we found are indeed protective or causative of DR. Finally, our patients did not show retinal structural and/or vascular changes, the latter finding further suggesting that these patients may be protected by clinical and subclinical modifications.

7 MATERIALS AND METHODS

7.1 Study participants

In this case-control study, Caucasian patients with type 1 diabetes for at least 30 years and without evidence or history of systemic microvascular complications (i.e. DR or diabetic nephropathy) were prospectively enrolled from the "Diabetes Research Institute" at the San Raffaele Scientific institute, Milan, Italy. The San Raffaele Ethics Committee approved this study which adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all patients prior to enrollment in this study.

Exclusion criteria for included patients were: (i) presence of DR as confirmed by ophthalmoscopy assessment, (ii) presence of any other retinal or optic nerve disease, (iii) history of previous ocular surgery, including intravitreal injection therapy, (iv) myopia superior to 6.00 diopters, (v) evidence of cataract, and (vi) evidence or history of uncontrolled systemic hypertension.

Patients' enrollment was completed between March 2022 and September 2022. Demographic and clinical information was obtained from the electronic medical record.

7.2 Whole-exome sequencing

After obtaining an IRB-approved consent, we obtained blood samples. DNA was extracted from 250 µL of blood using the DNA Kit named Maxwell® RSC (PROMEGA) using the Maxwell® RSC Instruments in order to provide an efficacious purification of genomic DNA. A novel paramagnetic particle (i.e. MagnaCel™) was used by this kit to purify samples. This particle allows for an efficient sample capture, as well as washing and purification of genomic DNA. Once the DNA was extracted, it was stored at -80 degrees Celsius before we were ready to perform sequencing.

To perform whole-exome sequencing (WES) analysis, we used Agilent SureSelect-Human-All-Exon V.6 probes to capture libraries and they were successively run on NextSeq 500 platform (Illumina, San Diego, California, USA). Raw reads were successively aligned to the reference human genome sequence (GRCh38/hg38) using bwa-mem2 software, the accelerated version of bwa-mem algorithm in bwa aligner. Duplicates reads were marked using MarkDuplicates tool from Picard tools and recalibration was performed with BaseRecalibrator to correct for technical biases.

For germline short variant discovery (SNPs + Indels), GATK v4.1.9 best practices were followed. HaplotypeCaller in GVCF mode was employed for germline variant calling. Next, GVCFs from multiple samples were consolidated into a GenomicsDB datastore. Joint genotyping was performed using GenotypeGVCFs, and finally, filtering was performed using VariantRecalibrator and applyVQSR to produce the final multisample callset with the desired balance of precision and sensitivity.

SelectVariants was employed to keep in the analysis only PASS variants. Variant annotation was performed using SnpEff v4.3 tool which includes annotations from Clinvar database, dbnsfp, dbsnp and GnomAD v3.

FastQC was used to qualitatively control FASTQ files (http://www.bioinformatics.babraham.ac.uk/ projects/fastqc/) (Figures 2-3).

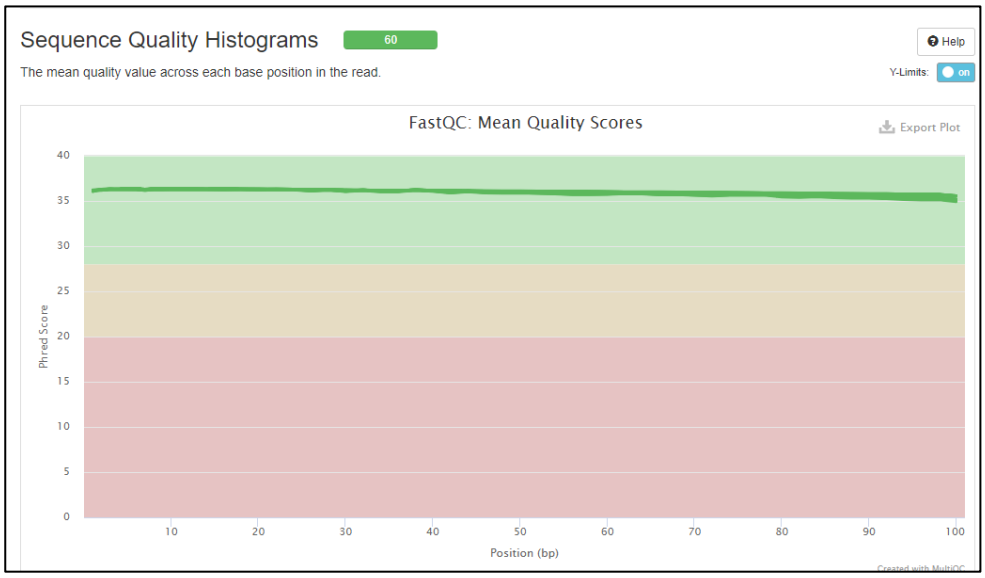

Figure 2. FastQC sequence quality histograms providing quality scores per base.

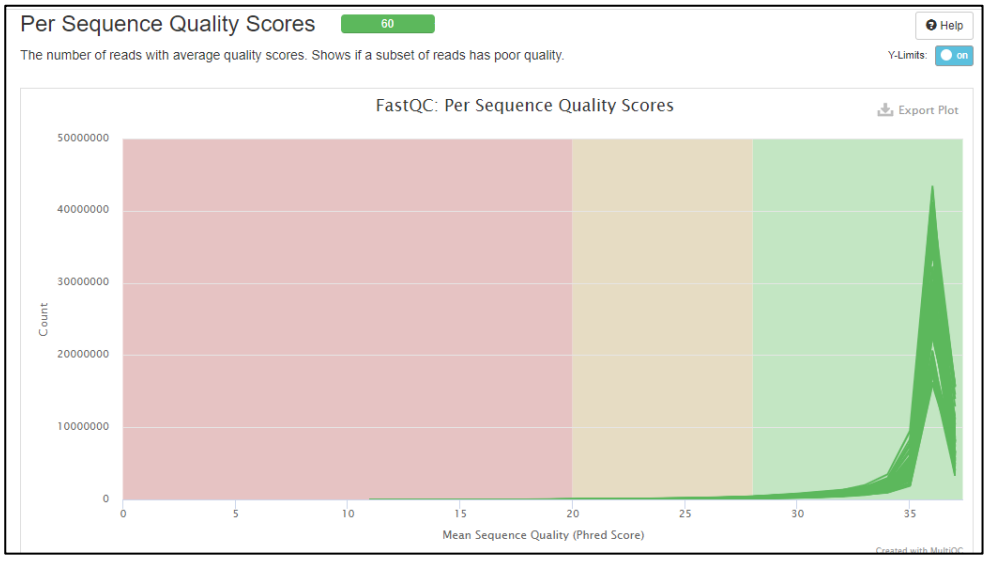

Figure 3. FastQC sequence quality histograms providing quality scores per sequence overall.

CollectHsMetrics by Picard tools was then used to assess the quality of alignments (Figure 4).

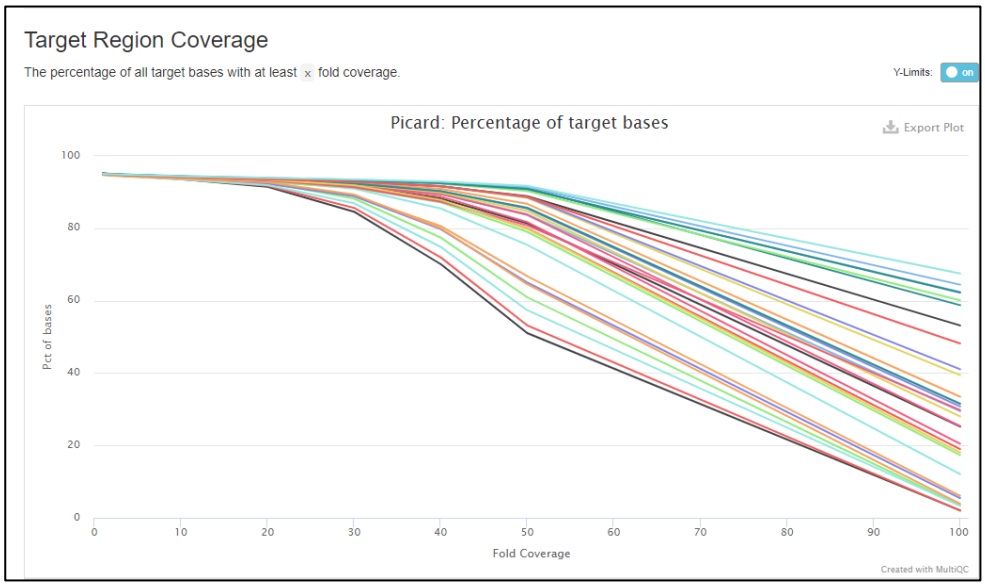

Figure 4. Graph showing genome coverage using Picard tools.

The total number of reads vary from a minimum of 54,913,796 to 142,964,976 reads. We report a median coverage of at least 80X [50-120]. Despite the heterogeneity, the coverage is enough to perform variant calling analysis for all the samples, with a median percentage of target exons captured at 30X over 90% [85-93].

The WES analysis was divided in three parts:

1. Step one – Given that our study cohort was relatively small, we sought to determine whether in our study cohort there were variants in well-studied genes that previous studies had suggested to be associated (i.e. either protective or causative) with diabetes-associated microvascular complications.
2. Step two – We did investigate presence of causative variants our study cohort.
3. Step three – We did investigate presence of protective variants our study cohort.

For steps 2 and 3, a control group of three patients with a diagnosis of proliferative

diabetic retinopathy (PDR) after a history of type 1 diabetes lasting less than 20 years was also included in the WES analysis for comparisons.

For step two, variants were filtered in order to include only those with a minor allele frequency less than 0.1%. Moreover, we selected only those variants that were likely to alter protein function (e.g. missense or loss-of-function mutations). Finally, we selected variants that were present in at least 2 out of three subjects in heterozygosis or homozygosis.

For step three, we selected variants that were absent in patients with PDR and short history of type 1 diabetes. Moreover, we selected only those variants that were likely to alter protein function. Finally, we identified candidate genes whose alleles were carried in as many subjects without DR as possible (at least 34 out of 54 alleles – allele count [AC] of at least 34).

7.3 C-peptide analysis

Enzyme-linked immunosorbent assay was used to measure serum C-peptide (C-peptide ELISA; Mercodia), as previously described (Rudovich *et al*, 2004).

Two age-matched control groups of thirty healthy subjects and thirty patients with PDR were included for comparisons. All control subjects had no evidence or history of systemic diseases.

7.4 OCT and OCTA imaging

Individuals with long-lasting type 1 diabetes and without systemic microvascular alterations also underwent a complete ophthalmologic examination, including structural OCT and OCTA imaging. The right eye was included in the analysis if both eyes were eligible.

The Heidelberg Spectralis HRA+OCT (Heidelberg Engineering, Heidelberg, Germany) device was employed to perform structural OCT. A volumetric scan covering the macula with 19 B-scans was performed, as previously described (Borrelli *et al*, 2021c). As recommended by the manufacturer,[15] we required a minimum signal strength of 25 to the OCT images to be included. Successively, the Spectralis built-in software was employed to automatically measure the inner retinal thickness within the foveal circle (dimensions: diameter of 1 mm) and parafoveal anulus (dimensions: diameters of 1 and 3 mm for the inner and outer circles, respectively) of the ETDRS-grid centered over the fovea, as previously described (Borrelli *et al*, 2021c).

The PLEX Elite 9000 device (Carl Zeiss Meditec Inc., Dublin, CA, USA) was employed to obtain swept source (SS)-OCT and SS-OCTA imaging by performing a 3x3-mm scan (300 A-scans x 300 B-scans) covering the macula. OCTA scans with a signal strength index (SSI) lower than 7 were excluded.

Analysis of OCTA images was done as previously described (Hirano *et al*, 2019; Chu *et al*, 2020). In brief, *en face* OCTA images segmented at different levels (i.e. SCP, DCP, and CC) were first exported from the device and then imported into Fiji ImageJ software version 2.0.0. Previously validated algorithms were used to binarize images and calculate the SCP and DCP perfusion density (PD) (Borrelli *et al*, 2021d). Subsequently, the binarized images were skeletonized to obtain the vessel length density (VLD). SCP and DCP metrics were investigated in an annular region of interest (i.e. parafoveal region; dimensions: diameters of 1 and 3 mm for the inner and outer circles, respectively). The percentage of flow deficits (FD%) in the CC image was calculated after applying the Phansalkar threshold, as previously described (Mastropasqua *et al*, 2019; Battista *et al*, 2020; Borrelli *et al*, 2020b, 2019c, 2019a;

Byon *et al*, 2019; Nassisi *et al*, 2018; Borrelli *et al*, 2020c, 2019d, 2017, 2021b). The CC FD% was separately measured in the foveal and parafoveal regions.

An age-matched control group of thirty healthy subjects undergoing structural OCT and OCTA imaging was included for comparisons. All control subjects had no evidence or history of ocular diseases. In control subjects, only the right eye was enrolled.

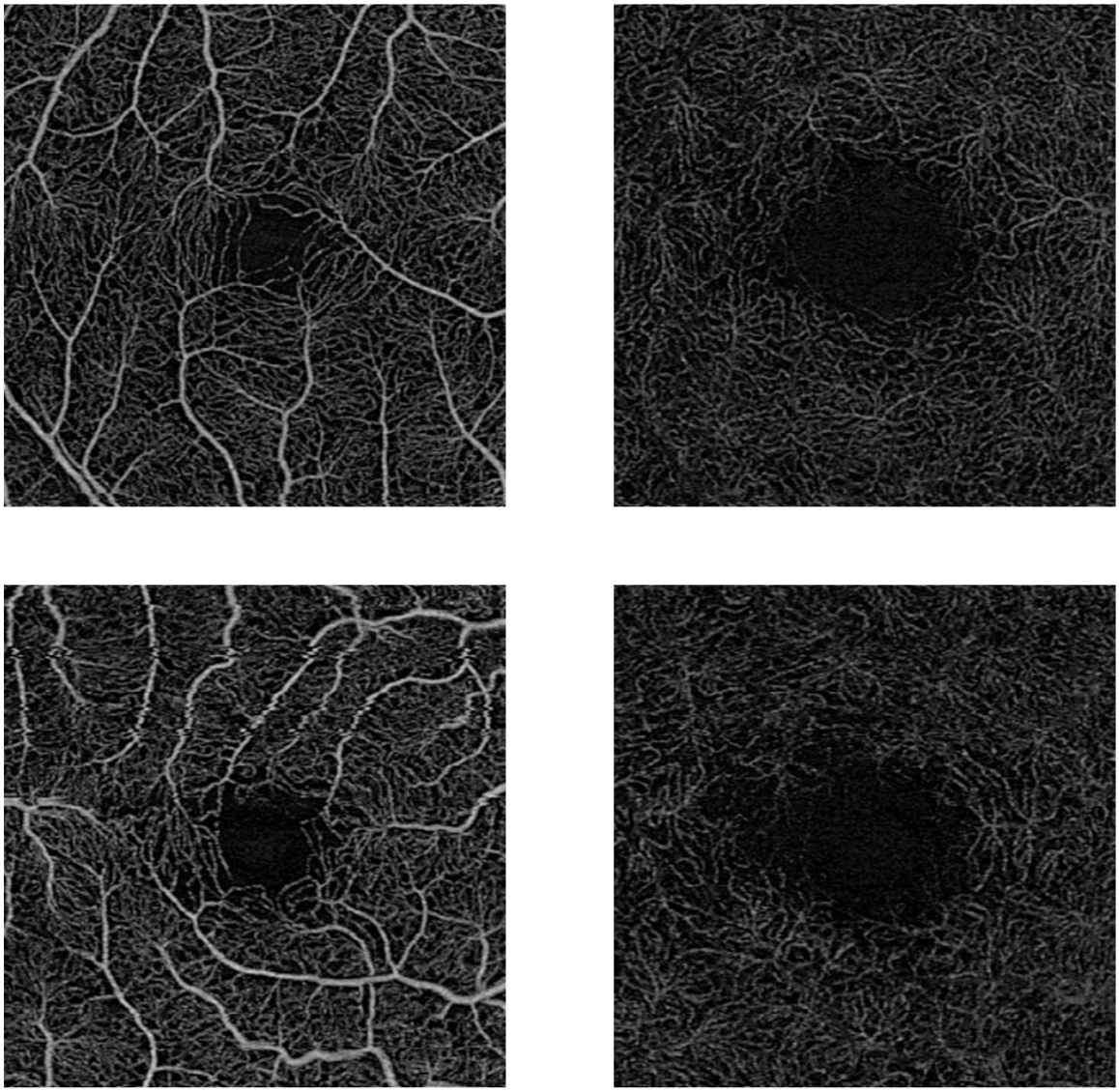

Figure 5. Representative OCTA images of the SCP (left column) and DCP (right column) from a diabetic patient (top line) and healthy control (bottom line).

7.5 Statistical analysis

The Statistical Package for Social Sciences (version 20.0, SPSS Inc., Chicago, IL, USA) software was used for statistical calculations.

To test normal distribution, the Shapiro-Wilk's test was used. A one-way analysis of covariance (ANCOVA) with age as covariate was employed to compare groups. A value of 0.05 was chosen for statistical significance.

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