Mitochondrial haplogroups in association study with onset and progression of diabetic retinopathy

Veronika Medova, [1,2] Andrea Soltysova,[1,2] Nikola Pribulova,[1] Ludevit Kadasi,[1,2] Andrej Ficek[1*]

[1]Department of Molecular Biology, Faculty of Natural Sciences, Comenius University, Ilkovicova 6 Bratislava, 842 15 Slovakia

[2]Institute for Clinical and Translational Research, Biomedical Research Centre, Slovak Academy of Sciences, Dubravska cesta 9, 845 05 Bratislava, Slovakia

***Corresponding author**

Word count: 3247 words (Title page and Abstract excluded)

Funding Information: The authors acknowledge the funding provided by Research and Development Operational Programme ITMS 26240120038, 2013/4.1/04-SORO.

Commercial relationships disclosures: None

Abstract

Purpose

Diabetic retinopathy (DR) is the most frequent microvascular complication in patients with diabetic mellitus (DM). Excessive formation of reactive oxygen species and mitochondrial dysfunction in retina suggest the possible role of mitochondrial variability on DR. We aimed to test for association of mtDNA haplogroups with occurrence and progression of DR in 361 Slovak diabetic patients.

Methods

3897 diabetic patients were included in the project in which clinical ocular examination was carried out for all participants. 361 patients, based on the presence of DR (G-RET - DR present within first 7 years since DM diagnosed, G-CON – no signs of DR after 17 years of DM) were selected for mtDNA haplotype determination by HV1 region sequencing to test for association with DR occurrence and progression.

Results

Based on clinical examination in 3897 patients we observed strong association of retinopathy with type 1 DM (p=0.00001) and association with type 2 DM when development of retinopathy within first 7 years of diabetes was considered (p=0.005). While no difference of DR occurrence was observed between males and females, strong association of DR with males was identified in G-RET group (p=0.0001). While mitochondrial haplogroup distribution did not significantly differ between G-RET and G-CON groups, indication of association of haplogroup HV (p=0,044) and M (p=0,033) with severity of DR was observed. This observation need to be replicated in further studies due to small sample size, however.

Conclusions

Mitochondrial haplogroups HV and M are supposed to be implicated in DR progression with further studies needed.

Keywords: diabetic retinopathy, mtDNA haplogroups, mitochondria

Introduction

Diabetic retinopathy (DR) is one of the most common complication of *diabetes mellitus* (DM) and is leading cause of vision loss in diabetic patients and working adults in developed countries. Prevalence of any type of diabetic retinopathy, vision threating progressive forms of proliferative diabetic retinopathy (PDR) and diabetic macular edema (DME) in diabetic patients is as high as 34,6%, 7,0% and 6,8%, respectively.[1]

Increased circulation of blood glucose through vessels during diabetes results in micro and macro vascular damage,[2] which is amplified by hypertension and dyslipidaemia as risk factors associated with arise and progression of the disease.[3,4] Multiple biochemical pathways and cellular mechanisms might explain diabetes driven complications, with some of them being studied the most like polyol pathway flux, increased advanced glycation end-products (AGEs) formation, protein kinase C activation signaling pathways or increased oxidative stress, reviewed in Sharma et al., (2019).[5] Ongoing inflammation, vascular occlusion and oxidative stress upregulate factors like vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), angiopoietins (Ang-2), tumor necrosis factor (TNF) and lead to progression of diabetic retinopathy.[1] Hyperglycaemic milieu creates conditions for increased glucose auto-oxidation and/or initiation of metabolic abnormalities which leads to formation of reactive oxygen species (ROS) thus creating excessive free radicals environment which is supposed to have the central role in the pathogenesis of retinopathy.[6]

Mitochondria is a significant source of reactive oxygen species which production is elevated in hyperglycaemia and resulting in damage to macromolecules and mitochondria dysfunction.[7,8] Genetic variability of mitochondrial DNA, driven by higher mutation rate in

mitochondria and defined in distinct matrilinear mtDNA haplogroups, is assumed to be phenotypically mostly neutral, however there have been several studies that shown association of certain mitochondrial haplogroups with various complex disorders.[9,10] Considering the role of mitochondria in diabetic retinopathy there have been published studies such as Bergman et.al. (2017), who described connection of haplogroup H to severity, but not prevalence of diabetic retinopathy, or Mitchell et al. (2017), who stated modifying effect of mitochondrial haplogroups U and UK on proliferative diabetic retinopathy in patients with type 2 diabetes.[11,12] On the other hand, one of the latest studies declared, that haplogroup H have no association with diabetic retinopathy in large Caucasian sample.[13]

The opposite findings of these studies led us to test the distribution of mtDNA haplogroups, based on HV1 region sequencing, in 361 Slovak patients with type 1 (T1DM) and type 2 (T2DM) diabetes considering the type of diabetes, sex, disease duration and degree of retinal damage.

Material and methods

Patient samples

During DIARET project (ITMS: 26240120038) 3897 diabetic patients' samples were collected and deposited in DNA bank with deidentified clinical data. Of these patients, two smaller clinically distant groups of patients were selected for further genetic study based on retinopathy occurrence in relation to diabetes duration. The first group (G-RET) consisted of 129 patients with diabetic retinopathy developed in less than seven years after DM diagnosis, while the second group (G-CON) consisted of 232 patients with diabetes duration for at least 17 years but no signs of diabetic retinopathy so far.

The ophthalmological examination was performed at the time of the project for all patients enrolled in the study. Considering the degree of retinopathy progression, NPDR was

considered as mild when one or both eyes were affected with presence of microaneurysm and/or retinal hemorrhages, while severe NPDR was considered when presence of more than 2 indicators (microaneurysm, hemorrhages and soft exudates, extensive intra-retinal hemorrhages in 4 quadrants, intra-retinal microvascular abnormalities and/or phlebitis in more than 1 quadrants) were present at least on one eye. PDR and DME were considered as severe complications.

The study was conducted according to the principles outlined in the Declaration of Helsinki and written informed consent was obtained from all patients.

Mitochondrial haplogroup identification

Mitochondrial hypervariable region I was amplified using HOT FIREPol® DNA Polymerase (Solis BioDyne, Estonia) and specific primers (upon request). Sequencing analysis of amplicons was performed using BigDye™ Terminator v3.1 Cycle Sequencing Kit and fragments were analysed using ABI Prism 3130xl Genetic Analyzer (Life Technologies, USA). Alignment to the reference sequence (NCBI NC 012920) and variants identification allowed for haplogroup assignment using Haplogrep2 classification tool.[14]

Statistical analysis

Chi-square test was used to analyze characteristics of the whole set of 3897 diabetic patients considering sex, age, type of diabetes in relation to retinopathy progression and diabetes duration. Chi-square test was applied also for comparisons of mitochondrial haplotypes and/or haplogroups distribution between selected two clinically distant groups of patients.

Results

3897 diabetic patients (53,3% women) of slovak (93%), hungarian (5.9%), czech (0.49%), and roma (0.33%) nationality with deindentified clinical data were included in the project. 10,09% of patients suffered from type 1 diabetes with the average age of diabetes onset and duration of disease at the time of the study 25.9/14.78 years, while in type 2 diabetes patients it was 53.19/8.47 years, respectively.

For the subsequent genetic analysis we selected two groups of patients, denoted G-RET and G-CON, depending on the presence of DR in relation to the diabetes duration. 129 (33,3% women) patients in G-RET group were characterized with maximum of 7 years from DM diagnosis (average 4.29y) and presence of retinopathy, while 232 (57,14% women) patients in G-CON group suffered from diabetes for minimum of 17 years (average 22,5y) without any clinical signs of DR (Table 1).

In the whole group of patients diabetic retinopathy was found to be significantly more often in T1DM patients ($p<0.00001$), and with reverse association observed after G-RET/G-CON selection ($p<0.0005$). Diabetic retinopathy was not associated with gender in all patients, however selection based on disease duration and retinopathy prevalence (G-RET/G-CON) showed diabetic retinopathy to be present more often in males ($p<0.0001$) (Table 1).

Table 1. Prevalence of diabetic retinopathy in relation to DM diagnosis duration and gender

	All patients N (%)					
	T1DM	**T2DM**	**p- value**	**Female**	**Male**	**p- value**
DR	128 (34.87)	529 (16.2)	**0.00001**	363 (17.46)	355 (19.53)	0.096
Without DR	239 (65.13)	2738 (83.8)		1716 (82.54)	1463 (80.47)	
	Selected groups of patients depending on extreme values					
G-RET	9 (15)	120 (39.87)	**0.0005**	43 (24.57)	87 (46.77)	**0.0001**
G-CON	51 (85)	181 (60.13)		132 (75.43)	99 (53.23)	

Based on observed mitochondrial HV1 sequence variation in 361 analyzed patients we identified 161 haplotypes belonging to 56 subhaplogroups (Fig. 1). Due to low sample numbers in groups we combined identified haplotypes into 10 common subhaplogroups. The most common haplogroup H allowed us to analyze samples of this group in separate H1 (8.86%), H2 (18.56%), HV (8.31%) and other H (15.24%) subhaplogroups. The rest of haplotypes belonged to subhaplogoup UK (19.95%), T (9.97%), J (7.2%), joined N-others (I, W, X, Y, N) (6.65%), M (3.32%) and R-others (R8, R9, R14) (1.94%). Distribution of these 10 haplogroups did not show any statistically significant (chi-square) difference between G-RET/G-CON groups (Fig.2A).

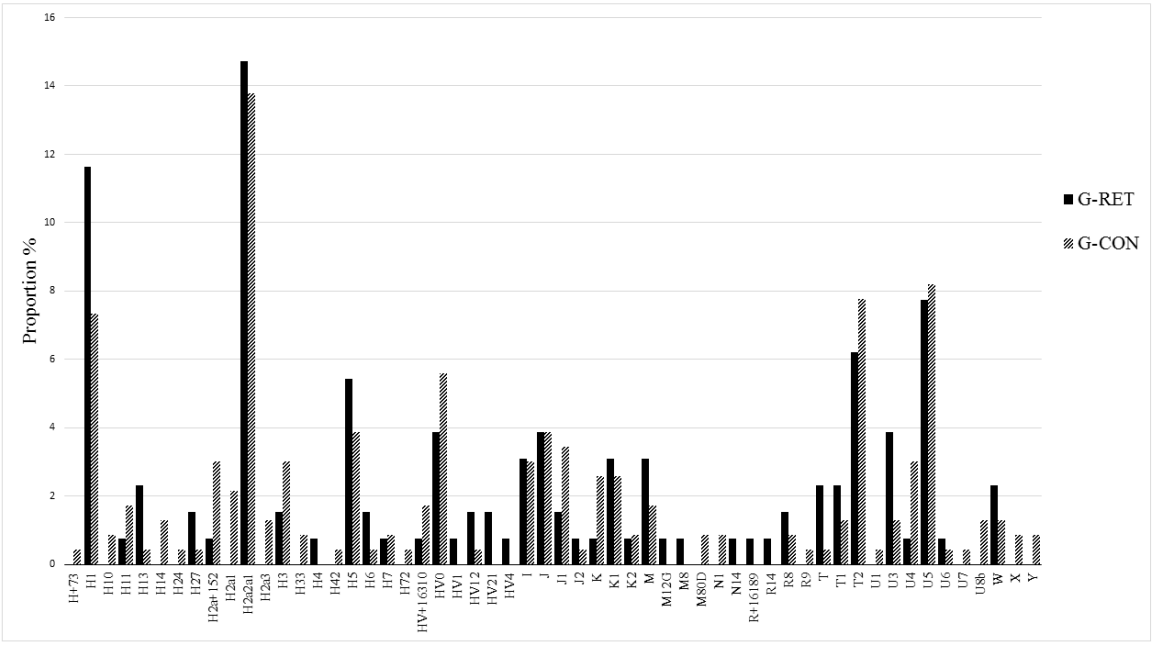


Figure 1. Occurrence of 56 mitochondrial subhaplogroups identified in 361 DM patients divided into two groups: G-RET - patients with retinopathy within 7y of DM, and G-CON - control group of DM without retinopathy after 17y of DM.

Since we observed association of sex with prevalence of the retinopathy in G-RET/G-CON groups, we tested for the association of mitochondrial haplogroups with DR status within individual sex groups separately. None of analyzed haplogroups was significantly associated with presence of DR complications in relation to sex.

Further, we tested haplogroup distribution for association in relation to the severity of DR. Patients with DR were divided into 2 groups based on severity of retinopathy, considering clinical presentation of retinopathy according to clinical ocular examination at the time of the study described in material and methods section. Similar distribution of haplogroups with no statistical difference in both (severe, mild) groups were observed, except for HV and M haplogroups (p=0,044, and p=0,033, respectively) which indicate the possible association of HV and M haplogroups with DM progression (Figure 2B).

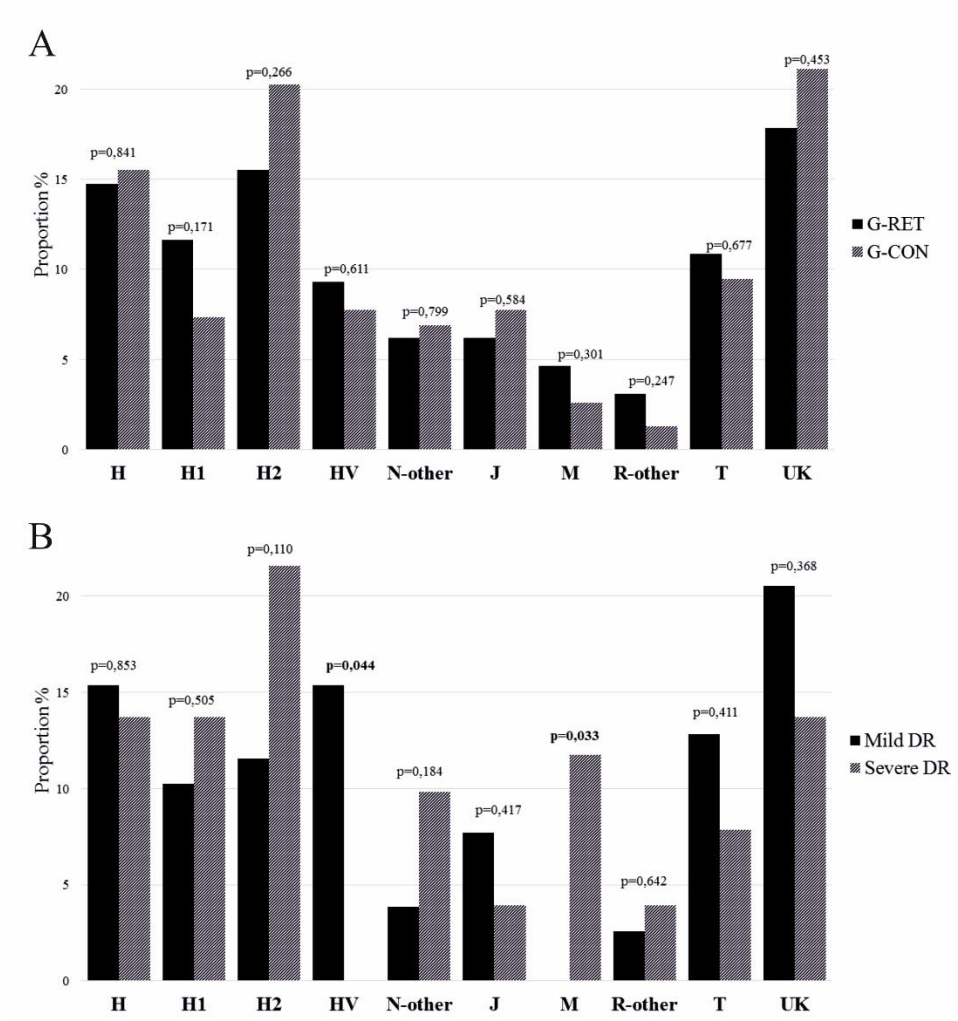


Figure 2. Proportion of observed haplogroups in retinopathy G-RET (black) and control - without retinopathy G-CON (hatch black) groups of DM patients (A), and proportion of observed haplogroups in G-RET patients divided into mild (black) and severe (hatch black) DR subgroups (B). P values from chi-square test are presented, with significant p values bolded.

Discussion

Diabetic retinopathy is one of the most common causes of acquired blindness in middle age population with prevalence to vary between 10-61% in patients with known diabetes and between 1.5-31% in newly diagnosed diabetes in various populations.[15] Sex-gender differences in the onset of diabetic complications, such as DR, seem to be independent risk factor according some studies, while others showed no statistically significant

difference.[16-18] The disease heterogeneity, selection criteria, number of patients analyzed, and etnicity in various studies may be responsible for such conflicting observations. Prevalence of DR in group of 3897 patients present in this study was 18.4% with observed difference between sexes only when considering the onset of DR with respect to the duration of diabetes. The presence of DR in male and female patients did not reveal significant difference in all patients (Table 1), however strong association of DR with males was seen in patients with retinopathy diagnosed within 7 years since the DM onset ($p=0.0001$).

Various studies considered the prevalence of diabetic retinopathy in relation to the type of diabetes.[19] In our observation, diabetic retinopathy was found to be present significantly more often in T1DM (34.87%) patients compared to T2DM (16.2%) when compared in all 3897 patients ($p<0.00001$), which is in agreement with other studies, such as Matuszewski et al. (2020).[20] Interestingly, there was seen a strong reverse association after G-RET/G-CON selection ($p=0.0005$), showing that diabetic retinopathy in T1DM usually do not develop within first years of DM diagnosis, but later in decades.[21]

As a causal link between high glucose levels and metabolic abnormalities observed in DR are considered ROS, which originate at higher rate in elevated glucose environment. Damage to the retinal mitochondria during ROS driven pathogenesis of DR leads to decreased copy number of mtDNA, transcription of mtDNA encoded genes, and subsequent mitochondrial dysfunction.[22] While ROS are constantly produced by mitochondria and mitochondrial dysfunction is seen in DR pathogenesis, mitochondrial genetic variation became the subject for possible implication in the onset and progression of DR. Even though the association of mitochondrial haplogroups to various disorders has been studied for long time and to date many studies observed association of different haplogroups as risk or protective factors in pathogenesis of different diseases, studies focused on the association of mitochondrial haplogroups to the diabetic retinopathy are rare. Several studies linked diabetic

retinopathy with one or other haplogroup but the results are inconclusive. While Kofler et al. (2009) observed association of T haplogroup with CAD and DR in T2DM patients, Achilli et al. (2011) identified relationship between DR in T2DM and haplogroup H.[23,24]

In this study we attempt to assess the possible association of mitochondrial haplogroups to the onset and progression of DR in analysis of two clinically distinct groups of DM patients. Mitochondrial haplotypes were determined based on HV1 region sequences in 129 DM patients with DR developed within 7 years since DM diagnosis (G-RET group), and 232 DM patients without any signs of DR despite of at least 17 years of DM duration (G-CON group). In these 361 patients we identified 161 haplotypes belonging to 56 subhaplogroups. Due to low numbers in these subhaplogroups, samples were combined into larger, common haplogroups. As expected the most represented haplogroup was haplogroup H (51,97%), the most common haplogroup in Europe, which was identified in 51.16% of G-RET and 50.86% of G-CON group. This allow us to analyze samples belonging to H haplogroup in separate H1, H2, and HV sub-haplogroups. The rest of haplotypes belonged to other H, UK, T, J, N-other (I, W, X, Y, N), M and R-other (R8, R9, R14) haplogroups. None of haplogroups showed significant difference in distribution between the two groups, not even after stratifying samples by sex. These findings are in agreement to those in study by Liu et al. (2019) which was carried out on 2935 Caucasian DR patients with neither of haplogroups H1, H2, UK, K or JT being associated with any type of DR (NPDR, PDR or DME).[13]

In the study by Bregman et al. (2017) severity but not prevalence of the disease was associated with mitochondrial haplogroups when haplogroup H was associated to be a risk factor for and haplogroup UK to be protective against proliferative diabetic retinopathy (PDR) among Caucasian DR patients.[11] This study characterized connection between mitochondrial haplogroups, duration of diabetes and HbA_{1C}, and identified haplogroups as risk factors for proliferative DR. The assumption is that in cells of patients with haplogroup H there is

increased ROS production as result of high glucose level and / or reduced ability to manage elevated oxidation stress leading to vascular damage in retina and damage to the mitochondria. These effects could be worsen by long duration of diabetes but also due to metabolic memory phenomenon. This phenomenon describes the fact that mitochondrial damage could progress even after re-institution of good glycaemic control. Considering mentioned factors it can be assumed that patients with haplogroup H may be more sensitive to the effect of prolonged diabetes and poor glycaemic control.[12,25] To test the association of mitochondrial haplogroups with DR severity, we stratify DR patients into two groups based on severity of clinical retinal findings to mild and severe according to Alghadyan 2011.[26] Clinical ocular examination was carried out at the time of the study so we can exclude various evaluation criteria changing over time. Similar distribution of all haplogroups in (severe, mild) DR groups of patients were observed, except for HV and M haplogroups, for which indication of weak association predetermines HV as protective (p=0,044) and M as risk factor for severe retinopathy (p=0,033). However, we must point out the limitation of this indication that lie in the small sample size of patients in groups: HV (12 patients out of 78 in mild and 0 patients out of 51 in severe DR group) and M (0 out of 78 in mild and 6 out of 51 in severe DR group). As mentioned before by other studies, the problem with small numbers of patients identified for some haplogroups greatly limits the approach and depends on demographic factors, distribution of haplogroups, study design and sample size. Nevertheless, these observations might be considered and tested in further studies.

Acknowledgment

The authors acknowledge the funding provided by Research and Development Operational Programme ITMS 26240120038, 2013/4.1/04-SORO.

References

1. Safi SZ, Qvist R, Kumar S, Batumalaie K and Ismail ISB. Molecular mechanisms of diabetic retinopathy, general preventive strategies, and novel therapeutic targets. *Biomed Res Int*. 2014; 2014: 801269
2. Sarhangi N, Khatami F, Keshtkar A, Heshmat R, Atlasi R, Mohammadamoli M. The Role of Mitochondrial DNA (mtDNA) in the Development of Diabetic Retinopathy (DR): A Systematic Review. *Med Hypothesis Discov Innov Ophthalmol*. 2017; 6 (2): 30–38.
3. Kowluru RA, Mishra M. Contribution of epigenetics in diabetic retinopathy. *Sci China Life Sci*. 2015; 58:556–563.
4. Cheung N, Mitchell P, Wong TY. Diabetic retinopathy. *Lancet*. 2010; 10;376(9735):124-36.
5. Sharma A, Valle ML, Beveridge C, Liu Y, Sharma S. Unraveling the role of genetics in the pathogenesis of diabetic retinopathy. *Eye (Lond).* 2019; 33(4): 534–541.
6. Kowluru RA. Mitochondrial stability in diabetic retinopathy: Lessons learned from epigenetics. *Diabetes*. 2019; 68: 241–247.
7. Kowluru RA, Chan PS. Oxidative stress and diabetic retinopathy. *Exp Diabetes Res*. 2007; 2007: 43603.
8. Stuart JA, Brown MF. Mitochondrial DNA maintenance and bioenergetics. *Biochim Biophys Acta*. 2006;1757(2):79-89
9. Hahn A, Zuryn S. Mitochondrial genome (mtDNA) mutations that generate reactive oxygen species. *Antioxidants (Basel).* 2019;8(9):392
10. Marom S, Friger M, Mishmar D. MtDNA meta-analysis reveals both phenotype specificity and allele heterogeneity: a model for differential association. *Sci Rep*.

290 2017;7:43449
291 11. Bregman JA, Herren DJ, Estopinal CB, Chocron IM, Harlow PA, Warden C, Brantley
292 MA Jr., Samuels DC. Mitochondrial haplogroups affect severity but not prevalence of
293 diabetic retinopathy. *Invest Ophthalmol Vis Sci.* 2017; 58(2):1346-1351.
294 12. Mitchell SL, Neininger AC, Bruce CN, Chocron IM, Bregman JA, Estopinal CB,
295 Muhammad A, Umfress AC, Jarrell KL, Warden C, Harlow PA, Wellons M, Samuels
296 DC, Brantley MA Jr. Mitochondrial haplogroups modify the effect of diabetes duration
297 and HbA1c on proliferative diabetic retinopathy risk in patients with type 2 diabetes.
298 *Invest Ophthalmol Vis Sci.* 2017; 58(14):6481-6488.
299 13. Liu E, Kaidonis G, Gillies MC, Abhary S, Essex RW, Chang JH, Pal B, Daniell M,
300 Lake S, Gilhotra J, Petrovsky N., Hewitt AW, Jenkins A, Lamoureux EL, Gleadle JM,
301 Burdon KP, Craig JE. Mitochondrial haplogroups are not associated with diabetic
302 retinopathy in a large Australian and British Caucasian sample. *Sci Rep*. 2019;9(1):612.
303 14. Weissensteiner H, Pacher D, Kloss-Brandstätter A, Forer L, Specht G, Bandelt HJ,
304 Kronenberg F, Salas A, Schönherr S. HaploGrep 2: mitochondrial haplogroup
305 classification in the era of high-throughput sequencing. *Nucleic Acids Res.*
306 *2*016;44(W1):W58-63.
307 15. Ruta LM, Magliano DJ, Lemesurier R, Taylor HR, Zimmet PZ, Shaw JE. Prevalence of
308 diabetic retinopathy in Type 2 diabetes in developing and developed countries. *Diabet*
309 *Med.* 2013;30(4):387-98.
310 16. Cui Y, Zhang M, Zhang L, Zhang L, Kuang J, Zhang G, Liu Q, Guo H, Meng Q.
311 Prevalence and risk factors for diabetic retinopathy in a cross-sectional population-
312 based study from rural southern China: Dongguan Eye Study. *BMJ Open*. 2019; 9(9):
313 e023586.
314 17. Yin L, Zhang D, Ren Q, Su X, Sun Z. Prevalence and risk factors of diabetic

retinopathy in diabetic patients: A community based cross-sectional study. *Medicine (Baltimore)*. 2020;99(9):e19236.

18. Ozawa GY, Bearse MA Jr, Adams AJ. Male-female differences in diabetic retinopathy? *Curr Eye Res.* 2015;40(2):234-46.

19. Yau JWY, Rogers SL, Kawasaki R, Lamoureux EL, Kowalski JW, Bek T, Chen SJ, Dekker JM, Fletcher A, Grauslund J, Haffner S, Hamman RF, Ikram MK, Kayama T, Klein BEK, Klein R, Krishnaiah S, Mayurasakorn K, O'Hare JP, Orchard TJ, Porta M, Rema M, Roy MS, Sharma T, Shaw J, Taylor H, Tielsch JM, Varma R, Wang JJ, Wang N, West S, Xu L, Yasuda M, Zhang X, Mitchell P, Wong TY, Meta-Analysis for Eye Disease (META-EYE) Study Group. Global prevalence and major risk factors of diabetic retinopathy. *Diabetes Care.* 2012;35(3):556-64.

20. Matuszewski W, Baranowska-Jurkun A, Stefanowicz-Rutkowska MM, Modzelewski R, Pieczyński J, Bandurska-Stankiewicz E. Prevalence of Diabetic Retinopathy in Type 1 and Type 2 Diabetes Mellitus Patients in North-East Poland. *Medicina (Kaunas).* 2020; 56(4): 164.

21. Jenkins AJ, Joglekar MV, Hardikar AA, Keech AC, O'Neal DN, Januszewski AS. Biomarkers in Diabetic Retinopathy. *Rev Diabet Stud*. 2015; 12(1-2): 159–195.

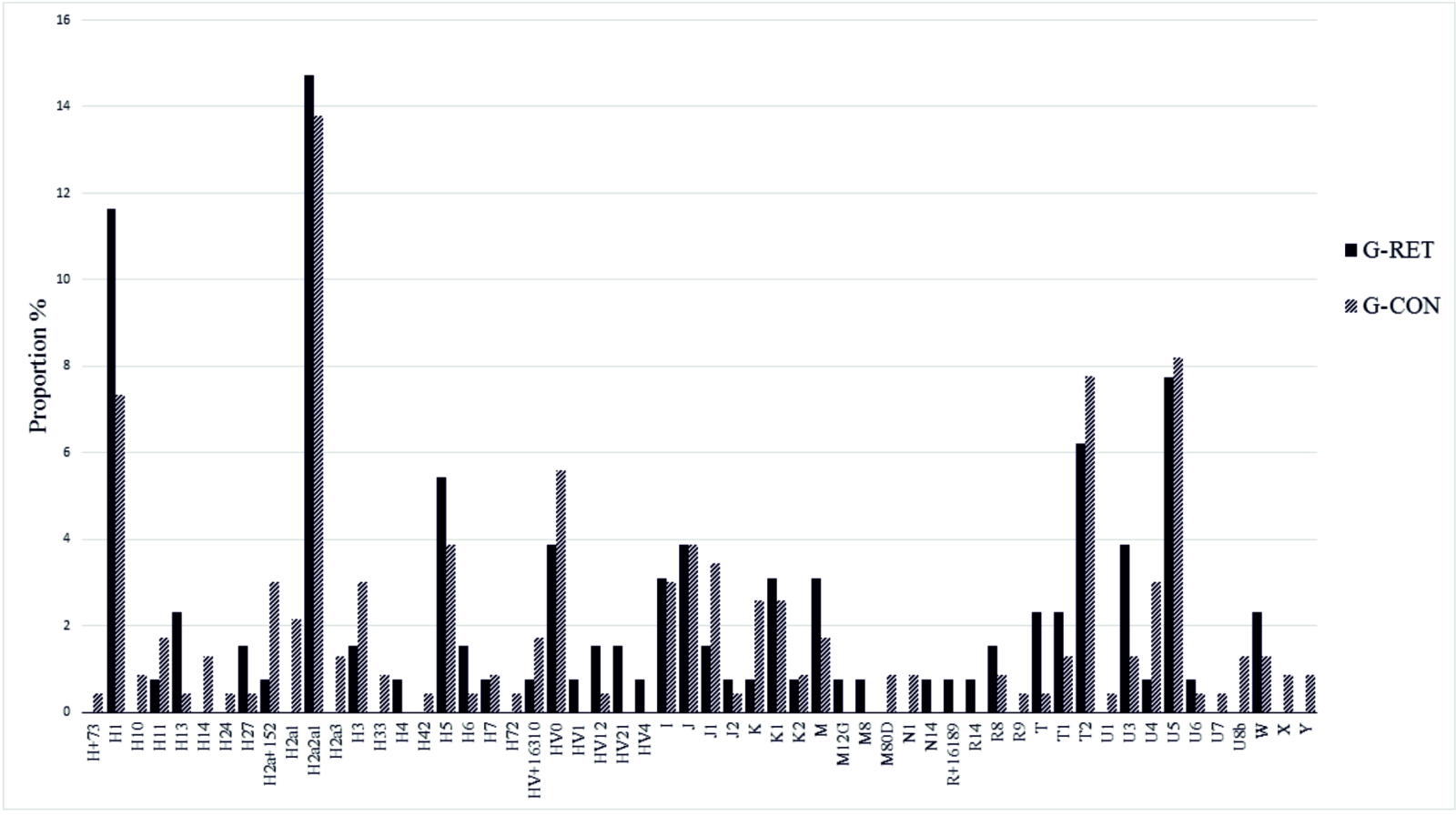
22. Massaad CA, Klann E. Reactive Oxygen Species in the Regulation of Synaptic Plasticity and Memory. *Antioxid Redox Signal.* 2011; 14(10): 2013–2054.

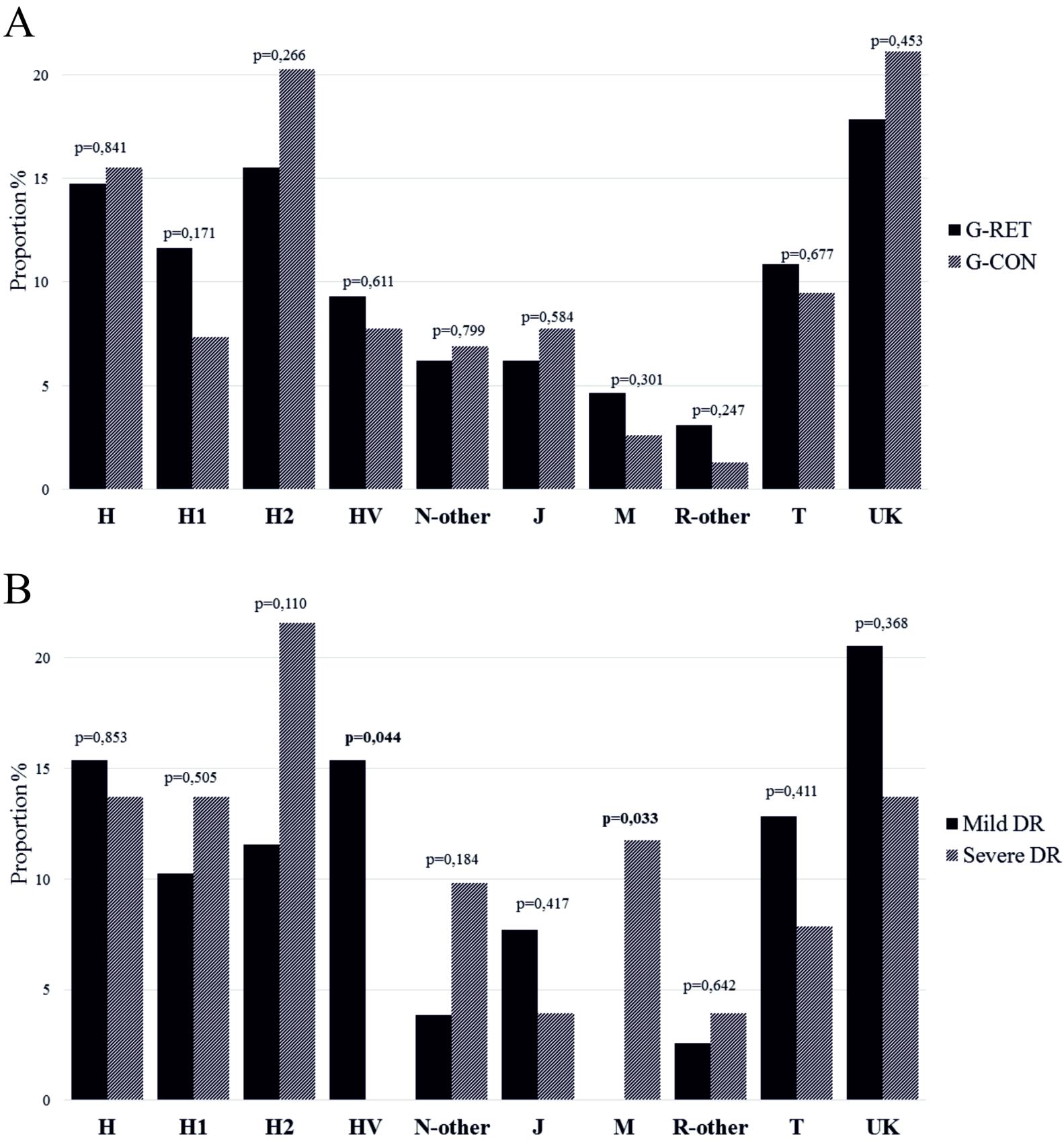
23. Kofler B, Mueller EE, Eder W, Stanger O, Maier R, Weger M, Haas A, Winker R, Schmut O, Paulweber B, Iglseder B, Renner W, Wiesbauer M, Aigner I, Santic D, Zimmermann FA, Mayr JA, Sperl W. Mitochondrial DNA haplogroup T is associated with coronary artery disease and diabetic retinopathy: a case control study. *BMC Med Genet*. 2009;10:35.

24. Achilli A, Olivieri A, Pala M, Kashani BH, Carossa V, Perego UA, Gandini F, Santoro A, Battaglia V, Grugni V, Lancioni H, Sirolla C, Bonfigli AR, Cormio A, Boemi M, Testa I, Semino O, Ceriello A, Spazzafumo L, Gadaleta MN, Marra M, Testa R, Franceschi C, Torroni A. Mitochondrial DNA backgrounds might modulate diabetes complications rather than T2DM as a whole. *PLoS One.* 2011;6(6):e21029.

25. Madsen-Bouterse SA, Mohammad G, Kanwar M, Kowluru RA. Role of mitochondrial DNA damage in the development of diabetic retinopathy, and the metabolic memory phenomenon associated with its progression. *Antioxidants Redox Signal*. 2010; 13(6): 797–805.

26. Alghadyan AA. Diabetic retinopathy – An update. *Saudi J Ophthalmol*. 2011; 25(2): 99–111.


Proportion %
16
14
12
10
8
6
4
2
0
G-RET
G-CON
H+73
H1
H10
H11
H13
H14
H24
H27
H2a+152
H2a1
H2a2a1
H2a3
H3
H33
H4
H42
H5
H6
H7
H72
HV+16310
HV0
HV1
HV12
HV21
HV4
I
J
J1
J2
K
K1
K2
M
M12G
M8
M80D
N1
N14
R+16189
R14
R8
R9
T
T1
T2
U1
U3
U4
U5
U6
U7
U8b
W
X
Y


A
Proportion %
20
15
10
5
0
p=0,841
p=0,171
p=0,266
p=0,611
p=0,799
p=0,584
p=0,301
p=0,247
p=0,677
p=0,453
H
H1
H2
HV
N-other
J
M
R-other
T
UK
G-RET
G-CON
B
Proportion %
20
15
10
5
0
p=0,853
p=0,505
p=0,110
p=0,044
p=0,184
p=0,417
p=0,033
p=0,642
p=0,411
p=0,368
H
H1
H2
HV
N-other
J
M
R-other
T
UK
Mild DR
Severe DR