SHORT TERM SCIENTIFIC MISSION (STSM) – SCIENTIFIC REPORT

The STSM applicant submits this report for approval to the STSM coordinator

Action number: BM1402
STSM title: Retinal pigment epithelium (RPE) in aging and in AD mouse models
STSM start and end date: 1/02/2018 to 30/03/2018
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PURPOSE OF THE STSM

Age-related eye conditions are the most common cause of sight loss and the rapidly increasing population of elderly people in Europe contributes largely to over 30 million blind and partially sighted persons in geographical Europe (www.euroblind.org). However, the etiology of age-related macular degeneration (AMD), a major cause of blindness among the elderly population is not known. Similarly, the Alzheimer's disease (AD) related visual impairment and its relation to AD pathology are not well understood.

The retinal pigmented epithelium (RPE) is a cell monolayer that is situated between the photoreceptors and the systemic circulation of the choroid and forms the outer blood-retinal barrier in the eye. RPE cells are highly specialized and active phagocytic cells that are necessary for the survival of the photoreceptors. Furthermore, the RPE polarity is responsible for the directional secretion of proteins, lipoprotein particles and lipid bilayer-enclosed extracellular vesicles (EVs). The results from a number of studies suggest that exosomes (part of EV) are not secreted merely as a degradation route for redundant molecules; rather they have specialized functions and play a key role in, among other things, intercellular signaling, and cellular waste management. Therefore, the RPE can be considered as the initial site of pathological changes in age-related macular degeneration (AMD) and possibly in AD related visual impairment.

The purpose of this STSM was the comprehensive analysis of the changes occurring in the RPE during 1. physiological aging (4 and 12 months old WT), and in 2. two different AD mouse models (5xFAD, 4 and 12 months old, and hAPPki 7 and 40 weeks old mice) and in Fish Oil (FO) treated WT and 5xFAD animals in order to reveal the potential mechanisms that can be targeted in order to restore the proper function of RPE. Unfortunately, the samples from FO-treated animals were degraded during the transport. However, we additionally analyzed the expression profile of genes involved in the cholesterol metabolism in RPE and retina and exosome markers in RPE of germ-free (GF) mice in order to understand the effects of the gut-retinal axis. All experimental groups were also analysed for the changes in the expression levels of the visual cycle genes.

DESCRIPTION OF WORK CARRIED OUT DURING THE STSMS

Animal models used in this project were 5xFAD mice (14 animals), hAPPki mice (16 animals), GF mice (10 animals) and WT mice (35 animals), both female and male. 5xFAD animals were 4 and 12 months old, hAPPki mice were 7 and 40 weeks old and GF mice were 8 weeks old. The total amount of 75 animals was used. These animals were also used for other experimental procedures and other organs, such as brain, liver etc. were dissected. The eyes were taken and RPEs, retinas and vitreous bodies were isolated. One eye from each animal was used for the RNA isolation for the qPCR analyses and the other eye was used either for the volume-SEM, or was fixed and afterwards processed for the histological analyses and immunohistochemistry.
In the host laboratory STSM applicant focused on gene expression analyses of the specific markers and molecular pathways as was proposed in the initial application:

1. Exosome markers – alix, cd63, tsg101 (APPki RPE, GF RPE)
2. Tight junction (TJ) genes – zo1, zo3, claudin, occludin (APPki RPE, GF RPE)
3. Genes involved in the regulation of cholesterol metabolism and unsaturated fatty acid transport – eaat2, cyp46, apoE, abca1, apoD, hmgcr, hmgcs1, srebp1a, srebp1c, srebp2, cyp27, lxrb, adipoR1, mfsd2a (APPki RPE and retina, GF RPE and retina)

In addition, the applicant analysed the changes in the battery of visual cycle genes under the regulation of sox9 –

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4. Visual cycle genes - sox9, otx2, crx, rpe65, lhx2, best1, ptgds, rbp1, rlbp1, rdh5, dct, lrat, rgr, mitf, tyrp1, tyr (5xFAD RPE, APPki RPE, GF RPE).

qPCR data was analysed in the qBASE program, graphed in PRISM and analysed further for the statistical significance.

The eyes used for histology were fixed overnight (ON) in 4%PFA and processed for paraffin embedding, cut on microtomes and stained with haematoxylin and eosin for further analyses. The eyes used for the volume-SEM were fixed in glutaraldehyde and osmium tetroxide ON and delivered to the Imaging Core for the further processing. The eyes for the immunohistochemistry (IHC) were fixed ON in 4%PFA and embedded in gelatin/sucrose and will be cut on cryostat and processed further for the immunohistochemistry.

The vitreous bodies from the eyes processed for the RNA extraction were isolated, homogenized in 1xPBS, rendered cell-free, frozen at -80C and will be analysed on Nanosight for the amount and size distribution of small vesicles.

DESCRIPTION OF THE MAIN RESULTS OBTAINED

A detailed analysis of qPCR data from the above mentioned groups of genes showed significant alterations in their expression levels in the RPE and in retina in both APPki and 5xFAD models and in the GF mice revealing the specific vulnerabilities that can be targeted .

The expression analyses of the genes regulating cholesterol metabolism in the RPE of APPki mice - srebp2 and hmgcs1 expression levels were significantly decreased in the 40w TG RPEs when compared to their age-matched WT controls, as was the expression level of cyp46 while cyp27 expression was increased in the same animals. On the contrary cyp46 was increased in the 7w TG RPEs. apoE expression was increased and abca1 and eaat2 expression was decreased in the 40w old TG RPEs (Fig.1).

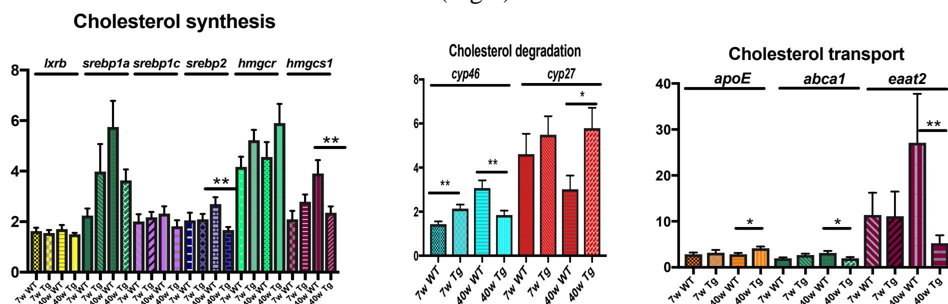

Figure1. qPCR analyses of the genes regulating cholesterol metabolism in RPE of APPki mice

The expression analyses of the genes regulating cholesterol metabolism in the retina of APPki mice – lxrb was increased in retinas of the 7w old TG mice. On the contrary the expression levels of srerbp1a was decreased in this age group although it was increased in the 40w old mice. The expression levels of hmgcs1 were decreased in the retinas of 40w old TG mice. The expression levels of the cholesterol degradation enzymes were unchanged except for the cyp46 whose expression was decreased in the 40w old animals. The expression levels of transporter genes was not altered except for the expression of eaat2 which was decreased in the retinas of 40w old TG mice (Fig.2).

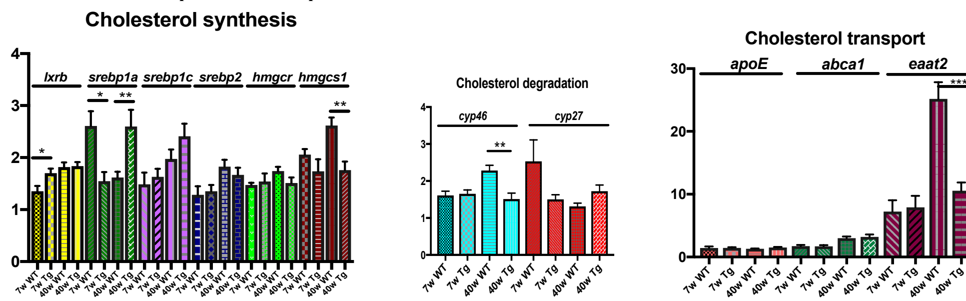

Figure2. qPCR analyses of the genes regulating cholesterol metabolism in retinas of APPki mice

The expression analyses of other genes analysed in retinas and RPEs of APPki mice - The DHA transporters expression was changed in the TG mice – adipoR1 expression was decreased in the RPE and unaltered in retina, and the expression of mfsd2a was not altered in either of the structure analysed.

From the TJ genes analysed, zo1 and occludin expression levels were decreased in the RPEs of the 40w old TG mice. Exosome markers analysed showed only the changes in the expression levels of alix, which was decreased in the RPEs of the 40w old TG mice. The analyses of the expression levels of the visual cycle genes showed that even without changes in the expression of sox9, several of these genes had altered expression in the RPEs od the 40w old TG mice. These genes (otx2, rpe65, lrat, best1, tyrp1, dct) showed decreased expression levels when compared to their age-matched WT controls.

The expression analyses of the genes regulating cholesterol metabolism in the RPE of GF mice – The expression levels of lrxb, srebp1c, srebp2 and hmgcr were increased in the RPEs of GF mice while the expression levels of cyp46 and cyp27 were not altered. apoE expression was decreased in the GF mice (Fig.3).

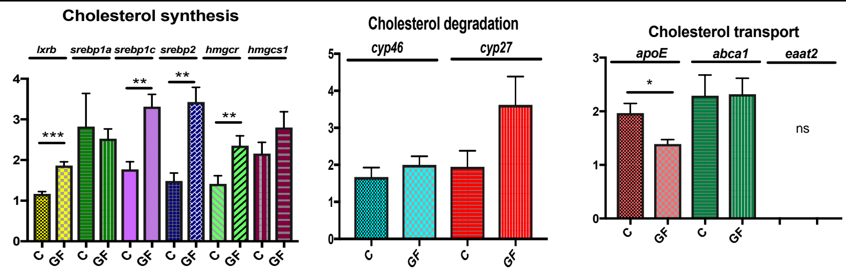

Figure 3. qPCR analyses of the genes regulating cholesterol metabolism in RPE of GF mice

<u>The expression analyses of the genes regulating cholesterol metabolism in retinas of GF mice</u> – was mostly unaltered in except for the expression of srebp1a and cyp46 which were decreased in retinas of GF mice (Fig.4).

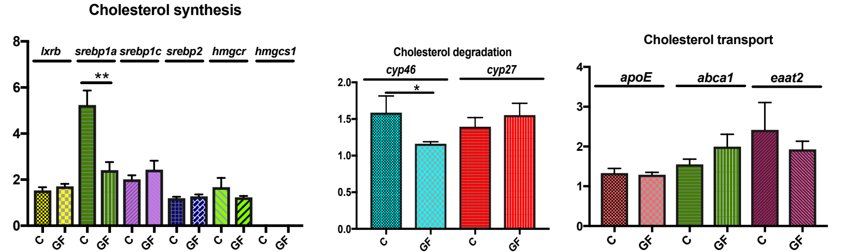

Figure 4. qPCR analyses of the genes regulating cholesterol metabolism in retinas of GF mice

<u>The expression analyses of other genes analysed in the RPEs and retinas of GF mice</u> The DHA transporters (both adipoR1 and mfsd2a) expression was increased in both RPEs and retinas of the GF mice. TJ gene expression was mostly unaltered in the RPEs of GF mice except for the expression levels of claudin14, which was significantly increased. Exosome markers analysed showed only the slight change in the expression levels of tsg101, which was increased in the RPEs of GF mice. The analyses of the expression levels of the visual cycle genes analysed showed that the expression of sox9 was increased and otx2 decreased and consequently the expression of all other visual cycle genes analysed except rbp1 and rgr was significantly decreased.

<u>The expression pattern of visual cycle genes in 5xFAD mice</u> showed no changes in the expression of sox9 and otx2. However, the expression levels of rpe65 and crx were increased in RPEs of 4m old 5xFAD mice, while the expression of rbp1 and rlbp1 was decreased in the RPEs of 5xFAD mice.

<u>Histological analyses</u> of GF and APPki eyes showed that there is no obvious difference between the control and GF mice. However, the RPE in 7 and 40w old APPki showed signs of deterioration (Fig.5).

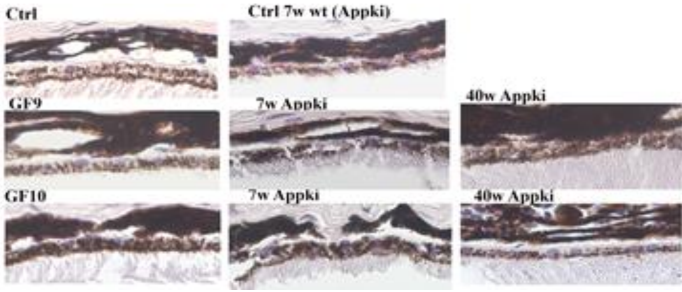

Figure 5. Haematoxylin and eosin staining of paraffin sections of the eyes from the control, APPki and GF mice.

<u>FUTURE COLLABORATIONS (if applicable)</u>

Part of the analyses will be continued in the Belgrade lab (Kanazir lab), specifically the analyses of the expression changes of the visual cycle genes during the physiological aging and in the aged 5xFAD animals. Furthermore, the sections from cryopreserved APPki and GF eyes will be stained for the retinal and RPE markers, and for the APP and Abeta expression. The volume-SEM analyses of GF and APPki mice is still on-going in the Vandenbroucke lab. We are planning to write 2 papers based on the data obtained during this STSM and on the follow-up data that will be produced afterwards in both labs.