The role of mitochondrial function and ABC transporters in healthy aging and Alzheimer's disease in the light of genetic modifications

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„Die Summe unserer Erkenntnisse besteht aus dem, was wir gelernt, und aus dem, was wir vergessen haben.“

Marie Freifrau von Ebner-Eschenbach

Contents

Abbreviations

ApoE	Apolipoprotein E
APP	Amyloid precursor protein
APP/PS1	APP/presenilin 1
ATP	Adenosine triphosphate
B6	C57BL/6J background
BBB	Blood-brain barrier
BCA	Bicinchoninic acid assay
BCSFB	Blood-cerebrospinal fluid barrier
BSA	Bovine serum albumin
com	common
COX	Cyclooxygenase
CTF	C-terminal fragment
DNA	Deoxyribonucleic acid
ddH2O	double-distilled water
dNTPs	Deoxynucleotides
ELISA	Enzyme Linked Immunosorbent Assay
EOAD	Early-onset Alzheimer`s disease
FAD	Familiar Alzheimer`s disease
for	forward
GFAP	Glial fibrillary acidic protein
GWAS	Genome wide association studies
HE	Haematoxylin and Eosin
IBA1	Ionized calcium binding adaptor molecule 1
IFNγ	Interferon γ
IL	Interleukins
KC/Gro	Keratinocyte chemoattractant/ human growth-regulated oncogene
ko	knockout
LOAD	Late-onset Alzheimer`s disease
MRI	Magnetic Resonance Imaging
MSA	Multisystem atrophy
MSD	Mesoscale Discovery
mtDNA	mitochondrial DNA
NeuN	Neuronal Nuclei
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	Parkinson`s disease
PET	Positron emission tomography
PFA	Paraformaldehyde
PSP	Progressive supranuclear palsy
rc	reverse complement
rpm	rounds per minute
RT	Room temperature
SNPs	Single nucleotide polymorphisms
SEM	Standard error of mean
Th1/Th2	T helper 1/ T helper 2
Thy1	Thymocyte antigen 1
TNFα	Tumour necrosis factor α
Ucp2	Uncoupling protein 2
wt	wildtype

Summary

Alzheimer's disease is a neurodegenerative disease characterized by progressive accumulations of neurofibrillary tangles, amyloid β plaques, and neuroinflammation. These neurotoxic accumulations, caused by intrinsic and extrinsic factors, lead to extensive neuronal loss and the progression of Alzheimer's disease. Even though this disorder is inherited in some families, the most common type is the sporadic form characterized by a late-onset. Mutations causing familiar forms of Alzheimer's disease have become powerful tools for its investigation. Especially, an aggressive double mutation discovered in a Swedish family in combination with other genetic modifications is helpful to characterize genetic and metabolic effects on Alzheimer's disease. Unfortunately, these mouse models often have different genetic backgrounds. For example, Scheffler et al. and Krohn et al. have shown that alterations of mitochondrial and ABC transporter function affect Aβ accumulation. While modified mitochondrial functions were investigated in APP-transgenic chimeric mice (B6mtFVB, B6mtAKR, B6mtNOD) and Uncoupling protein 2 knockout mice with C57BL/6J background, APP-transgenic ABC transporter knockout mice were examined in FVB background. It is well known that different genetic backgrounds have various effects on many disorders. So far, no detailed comparative study about the effect of genetic backgrounds C57BL/6J and FVB on the Alzheimer's disease mouse model APP/PS1 exists.

Therefore, one aim of this study was to check genetic background effects and additionally those of modified mitochondrial and ABC transporter function on aging and Alzheimer's disease pathology. For that reason, Aβ40 level, guanidine, and buffer soluble Aβ42 levels, cytokine levels, microglia coverage, astrocyte coverage and plaque development were analyzed.

Interestingly, none of the mentioned characteristics was found to be changed by genomic background strain, neither C57BL/6J nor FVB, not even with rising age. Comparing all mouse strains carrying either altered mitochondrial DNA or a knockout of Uncoupling protein 2 revealed that only the B6mtFVB chimerism causes changes in cytokine/chemokine levels i.e. IL-10 which might be caused by transcriptional or translational problems. Apart from that, mice with mitochondrial alterations do not show changes of the analysed factors during healthy aging. However, depending on genomic background and chimerism mice react different to the accumulation of amyloid β. APP-B6mtFVB mice display a preference to form new plaques and to inhibit plaque growth in comparison to APP-B6 and APP-FVB mice.

Simultaneously, the decreased mitochondrial membrane potential caused by a knockout of Uncoupling protein 2 led to several increased cytokine/chemokine levels. Out of all tested ABC transporter knockouts (ABCB1, ABCC1, ABCG2) only the knockout of ABCC1 increases immune response to Alzheimer disease significantly. With rising age astrocyte coverage increases in B6 and FVB background while microglia coverage rises in B6 background only.

These data clearly show that AD is indeed a multifactorial disease affected by distinct nuclear and mitochondrial genetic modifications. Moreover, this study proves that genetic backgrounds, mitochondrial alterations, and ABC transporter knockouts influence each other and give an explanation why it is so difficult to explore a cure for AD. On the other hand, they yield new information that might help to explain the genesis of AD. Further research is needed to elucidate the connections and mechanisms between AD pathology influencing factors like ATP-producing mitochondria and ATP-consuming ABC transporters.

1. Introduction

1.1. Alzheimer's disease – the most common neurodegenerative disease

With 60 – 80% of all cases Alzheimer's disease (AD) is the most common type of dementia. In 2015, 48 million people were suffering from AD. Until 2050 the number of neurodegenerative diseases will rise to 131.5 million and most types will be Alzheimer disease [1]. Since AD was described in 1906 for the first time by Alois Alzheimer, research has extended our knowledge tremendously. Alzheimer observed a patient named Auguste Deter and noted histological characterizations and several symptoms of AD. The most notable histological hallmarks are accumulations of proteins τ and amyloid-β (Aβ). The neurotoxic nature of these accumulating proteins causes cerebral atrophy. While τ forms intracellular tangles, Aβ accumulates extracellular in form of plaques surrounded by microglia. This close local relation between Aβ and immune cells shows that this characteristic protein of AD provokes a severe immune response.

The formation of plaques is an extracellular aggregation of Aβ, a protein generated by sequential enzymatic proteolysis of the amyloid precursor protein (APP) [2,3]. APP, a transmembrane protein, can be processed in a common non-amyloidogenic or frequent amyloidogenic pathway (Figure 1). In the non-amyloidogenic pathway α-secretases cleave APP within the Aβ region leading to a N-terminal soluble APP-fragment (sAPPα) and a membrane-bound C-terminal fragment (C83) which is cleaved further into the fragment p3 (24-26 amino acids) and the APP intracellular domain (AICD) by the γ-secretase. During the amyloidogenic pathway a cleavage by the β-secretase results in N-terminal soluble APP-fragment (sAPPβ) and a membrane-bound C-terminal fragment (C99). Further cleavage by γ-secretases results in AICD and a variety of Aβ peptides that can be 34 to 50 amino acids (Aβ34 - Aβ50) long. The imprecise cleavage causing this variety [3,4] is reviewed in Kummer et al. [5]. Although usually a small amount of Aβ42 is produced, its high propensity to aggregate, its hydrophobicity and its neurotoxicity lead to the progressive death of neurons and associated symptoms in AD patients (reviewed in Thinakaran et al. and Haass et al.[6,7]).

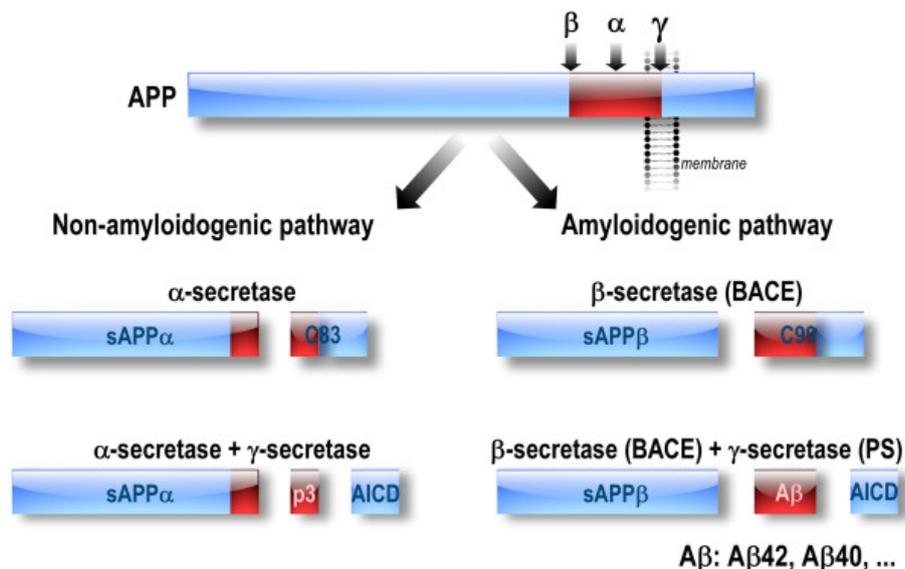**Figure 1: Non- and amyloidogenic pathways of APP processing**
Enzymatic proteolysis of APP by β- and γ-secretases generates variants of Aβ. (amyloidogenic pathway, right side). Contrary, Aβ is not produced during non-amyloidogenic processing by α- and γ-secretases (left) (adapted from Del Prete et al.[8]).

Degree and appearance of symptoms induced by neuronal death depend on the stage of this neurodegenerative disease. Beside memories patients gradually lose their ability to orientate in time and space. Additionally, patients can suffer agnosia and loss of motoric function. The first onset of symptoms in AD patients determines the disease type. If patients younger than 65 years show first symptoms, the disease is termed early-onset AD (EOAD), otherwise it is the more common sporadic type or late-onset AD (LOAD). 5-10% of all Alzheimer´s cases are EOAD and thirteen percent of those are familiar AD (FAD) cases, which are caused by inherited genetic mutations. Some known genetic mutations of FAD have been implemented in mouse models which are until today very helpful tools in the investigation of AD.

While the cause of FAD is well-known, the cause of LOAD is still unknown. Nevertheless, many risk factors have been identified. Aging is the most important one. While the prevalence of AD is 3% at the age of 65, it increases to 47% for persons older than 85 years [9]. Additionally, several studies discuss other potential risk factors like gender, familiar predisposition, smoking, head trauma, education, descent, cardiovascular diseases etc. [10-12]. Elimination of these risk factors is the best opportunity to prevent suffering from AD.

However, although research made great progress in the last years, it is still not possible to diagnose AD before first symptoms occur. A battery of neuropsychological tests (Mini-Mental State Examination, Alzheimer´s Disease Assessment Scale-cognitive etc.) and imaging methods (Magnetic resonance imaging, Computer-assisted tomography, Positron emission tomography etc.) are used to diagnose Alzheimer's disease [13,14]. While neuropsychological tests can indicate even mild cognitive impairment, it is still difficult to assure an AD diagnosis and to exclude all differential diagnoses, i.e. vascular dementia, making diagnostic imaging and post-mortem analyses necessary to determine the exact type of dementia. Imaging methods visualize cerebral atrophy, a hallmark of advanced AD. Even if AD is diagnosed ante-mortem, up to now researchers were not able to find a cure. Current treatment paradigms do not delay or stop progression of AD, but only relieve symptoms. Cognitive problems can be treated with acetylcholinesterase inhibitors (tacrine, rivastigmine, galantamine, donepezil) and N-Methyl-D-aspartic acid receptor antagonist memantine [15-18].

However, these drugs moderate cognitive symptoms but they do not treat depression, a common concomitant disease. Interestingly, Hofrichter et al. recently discovered a plant extract that is approved as a drug against depression and reduces plaque burden in AD mice. Furthermore, this 80% ethanol extract of St. John´s wort improves memory and enhances neuroinflammation by activation of microglia. These facts indicate a biochemical link between AD and depression which involves inflammation in cerebral tissue and ABC transporter function [19].

1.2. Effects of protective mechanisms in brain on the pathology of Alzheimer's disease

Due to the brain`s importance, several mechanisms for its protection exist. On the one hand, the immune system in cerebral tissue evolved special cell types to protect the brain i.e. microglia and astrocytes. On the other hand, two distinctive and tight membranes strictly control the transport of substances between blood and brain.

Extrinsic factors, i.e. traumatic brain injury, and intrinsic factors, i.e. accumulations of toxic proteins, cause inflammatory responses. The progressive nature of neurodegenerative diseases leads to a chronic neuroinflammation. The coexistence of chronic neuroinflammation and neurodegeneration has been described in several studies, especially the influence of

neuroinflammation on AD pathology [20,21]. One of the most obvious histological characteristics in brains of AD patients is that activated microglia surround Aβ plaques [22]. This close local association of microglia and the accumulation of Aβ indicates that this protein activates microglia.

Therefore, one focus of AD research is the chronic activation of microglia and its effects on the progression of AD. In investigations of such an age-associated disease it is important to know how aging affects healthy brains. Kohman et al. revealed that even in healthy mouse brains the number of activated microglia increases with rising age [23]. Subsequently this increase reduces the capability of hippocampal neurons to form new memories [24]. However, post-mortem analysed AD brains show even higher numbers of activated microglia. Aging also leads to significant morphological changes, i.e. fragmentation, loss of fine processes and swellings at the terminals of cytoplasmic processes [25,26].

Interestingly, some morphological characteristics of aged brains resemble those of AD brains [27]. These findings support even more the close connection between age and AD. However, the role of microglia in AD pathology is controversially discussed. It is common knowledge that microglia are activated by Aβ, a toxic protein which they also phagocytose [28-30]. This phagocytosis accompanied by degradation of Aβ is supposed to be beneficial. Contrary; it is assumed that chronic activation of microglia leads to a switch of phenotype that is detrimental and contributes to progression of AD.

The phagocytosis activity of microglia can be compromised by proinflammatory cytokines. For example, experiments on BV2 cells have shown that IL-1β suppresses phagocytosis [31]. Diverse studies have shown that neurodegenerative diseases like AD affect several cytokine levels. Indeed, IL-1β is one of a few cytokines that have been proven to be changed in AD patients by numerous *in vivo* studies. To be exact, IL-1β level is upregulated in AD [32,33]. A disadvantage of the method utilized to obtain these data is that cytokine levels of AD patients were measured in brain homogenates, as it prohibits the determination of cell type causing an increased secretion of IL-1β. On the other hand, changes of cytokine levels measured in *in vitro* experiments do not consider the complex networking of different cell types which influences the production and degradation of cytokines to each other. Furthermore, a disruption of tissue is necessary to collect cells for *in vitro* experiments. This always implies the risk to change circumstances that are essential to mimic an *in vivo* situation.

Recently Guillot-Sestier et al. and Chakrabarty et al. independently published data revealing that IL-10 has similar effects like IL-1β. Both labs used complementary approaches showing that the anti-inflammatory cytokine IL-10 worsens AD pathology and cognitive impairment in mouse models. While Guillot-Sestier et al. used IL-10 overexpressing APP/PS1 mice, the data of Chakrabarty et al. were obtained with IL-10-deficient APP/PS1 mice. Both research groups state that increased IL-10 levels cause an upregulated Apolipoprotein E (ApoE) expression. Moreover, they revealed that high levels of IL-10 and ApoE expression inhibit Aβ uptake by microglia [34,35].

Cytokines do not only interfere with cells but also with each other. Their network is very complex and consists of proinflammatory and anti-inflammatory cytokines. Some of them play a role in the differentiation of T cells to T helper cells 1 (Th1) or T helper cells 2 (Th2) [36,37]. Others are both pro- and anti-inflammatory and do not affect differentiation of T helper cells but have other functions, i.e. they influence endothelial cells and therefore the permeability of blood-brain barrier (BBB) [38,39].

The second protective mechanism are two tight membranes: the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB). They protect the brain against pathogens and toxic compounds. The blood-brain barrier consists of a monolayer of endothelial cells and encompassing

pericytes as well as astrocytes. Like neuroinflammation these barriers can be counterproductive in the context of a neurodegenerative disease. Weiss et al. report that specific membrane receptors and transporters facilitate active transport of nutrients from blood to brain and efflux of toxic compounds from brain to blood (reviewed in [40]). These active transports are important as they affect the clearance of proteins from the brain. Additionally, this organ does not have a lymphatic circulation that can support clearance processes. However, Louveau et al. discovered functional lymphatic vessels which have all the molecular characteristics of lymphatic endothelial cells and thus they may contribute to the clearance of brain [41]. Moreover, a glymphatic clearance pathway driven by Aquaporin 4 exists in the central nervous system. This pathway leads to the exchange of solutes between cerebral spinal fluid and interstitial fluid and plays an important role in neurodegenerative diseases and especially AD [42,43]. Iliff et al. showed that a lack of aquaporin 4 gene causes a reduction of Aβ clearance in mice [44].

Despite these lymphatic vessels, a decreased transport activity contributes to the development of a proteopathy. Accumulations of misfolded proteins cause most neurodegenerative diseases like for example Parkinson disease, Huntington disease etc. Most of these diseases are also age-related. Hawkes et al. revealed that drainage of small plaques to blood is age-related and it correlates with changes in capillary density [45]. For that reason, clearing mechanisms at the blood-brain barrier are new potential targets for prevention and treatment of several diseases.

1.3. ABC transporters- targets for treatment of Alzheimer's disease?

Active transporters are necessary to overcome the BBB, a structure of endothelial cells connected to each other by tight junctions. One type of active transporters, which reside at the BBB and BCSFB among other barriers, are ABC transporters. They tightly regulate and facilitate specific transport of small molecules in both directions. On the one hand, they actively transport nutrients from blood to brain. On the other hand, they export toxic compounds from brain to blood.

49 human ABC transporters are known and belong to seven subfamilies of ABC transporters (ABCA to ABCG), but only a few of them are associated to neurodegenerative diseases. Genome-wide association studies (GWAS) of Hollingworth et al. and Naj et al. revealed a connection between some ABC transporters of subfamily ABCA and AD. For example, an increased risk to develop AD is correlated to some variants of ABCA7 [46,47]. Additionally, a connection between small nucleotide polymorphisms (SNPs) and upregulated expression of ABCA7 and Apolipoprotein J was discovered [48]. Their encoded proteins have protective effects.

Additionally, numerous studies showed a correlation between decreased ABC transporter activity and increased incidence of neurodegenerative diseases. In 2008 Bartels et al. performed the first *in vivo* experiment to prove this link. Bartels et al. examined patients that suffered from Parkinson disease (PD), progressive supranuclear palsy (PSP) or multi-system atrophy (MSA) with [^{11}C]-verapamil PET. A common characteristic of these diseases is the accumulation of α-synuclein (αSyn). This investigation demonstrated a specific accumulation of [^{11}C]-verapamil in cerebral regions where investigated diseases start and thus a reduced ABCB1 function in these regions [49]. In 2012 Assema et al. published similar results for AD showing decreased ABCB1 function in patients suffering mild to moderate AD [50].

This finding is consistent with Aβ measurements obtained by Mawuenyega et al. Quantifications of production and clearance rates of Aβ40/Aβ42 demonstrated an impaired detoxification. To be exact, in AD patients Aβ clearance is 30 percent lower than in controls [51]. An *in vivo* examination by Krohn et al. disclosed a link between impaired functions of ABCB1 and ABCC1 and increased Aβ levels. In ABCC1-deficient APP/PS1 mice Aβ level is 12- to 14-fold higher than in APP/PS1 mice [52].

Moreover, Hartz et al. support the hypothesis that decreased ABC transporter function is rather a consequence than a cause of AD, as Aβ levels in brain were significantly reduced in *ex vivo* brain capillaries even after restauration of ABCB1 expression and its related transporter activity [53].

Metabolites, drugs, and nutrients floating through the blood system can affect ABC transporter activity. Most known drugs influencing ABC transporters are inhibiting their function. For example, verapamil, an ABCB1 inhibitor, is a common drug for migraine headache prevention and treatment of tachycardia. Additionally, ABCC1 inhibitors are used to increase the therapeutic effect of chemotherapeutic drugs. In some types of cancer, expression of ABCC1 rises with progression of cancer (reviewed in [54]). Contrary, Krohn et al. demonstrated the effectiveness of thiethylperazine, a drug activating ABCC1, as possible treatment against AD [52].

To sum up, previous studies have shown that decreased ABC transporter function worsens AD pathology and that activators of ABC transporters are a promising treatment strategy. ABC transporter function in turn consumes energy generated by two conserved adenosine triphosphate (ATP)-binding sites of ABC transporters. Therefore, ABC transporter function depends on the availability of ATP produced by mitochondria. The relation between altered mitochondrial function and ABC transporter function was previously discussed by Pahnke et al. [55].

1.4. Effects of mitochondrial alterations on Alzheimer's disease

Mitochondria are important for ABC transporter function and thus for AD as they generate ATP. Several reports have shown morphological, biochemical, and genetic abnormalities in AD tissues. So far, no specific mutation in mitochondrial DNA has been identified which causes these alterations. Based on these findings researchers assume that the morphology and biochemistry of mitochondria are altered because of translational mechanisms in AD. Certainly, it is not clear whether mutations in mitochondrial DNA are the cause or consequence of AD [56,57]. Moreover, it is possible that these changes are induced by rising age. For example, Heggeli et al. state that the increased risk in women to suffer from AD is caused, at least in part, by female longevity [58].

The enzymatic activity of proteins of the respiratory chain decreases with rising age [59]. Mutations in mtDNA cause this decline which is affected stronger by point mutations than by deletions [60]. The number of mutations in mtDNA increases with advancing age and is associated with neurodegenerative diseases [61,62]. Many published reviews discuss the fact that some mutations result in excessive production of reactive oxygen species in neurodegenerative diseases like AD and PD [63-65]. For instance, Bender et al. revealed that large deletions in mtDNA are linked to oxidative stress and cause neuronal death in PD [66]. Reactive oxygen species in turn cause cellular damage and mutations of mtDNA itself. Furthermore, it promotes altered mitochondrial function [67]. Accordingly, a vicious cycle is formed, and the resulting cellular dysfunction can lead to an altered ATP production. So, oxidative stress and mutations in mtDNA are the most important factors that play a role in the development of neurodegenerative diseases.

In AD, Aβ affects key enzymes of the respiratory chain, inhibits respiration, and alters ATP production [68-73]. Due to the complex interactions between energy-producing enzymes knowledge about key enzymes and respiration is limited. On the one hand, it was described that Aβ binds subunit one of the cytochrome c oxidase (COX) and hence induces its inhibition [71]. Kim et al. demonstrated that Aβ triggers a release of COX and a swelling of mitochondria in neurons which activates caspase-3 and finally leads to apoptotic cell death [74]. Moreover, Fukui et al. revealed that cytochrome deficiency leads to a downregulation of ROS production and Aβ accumulation, while the expression of APP remains stable [75]. Overall, these contradictory results illustrate how little is known about the role of COX in AD pathology.

Uncoupling proteins (Ucp) are mitochondrial inner membrane proteins acting like proton carriers between matrix and intermembrane space. In mammals five different types are known (Ucp1 – Ucp5). Ucp knockouts lead to increased proton levels in the intermembrane space. Previous research focused on disclosing the role of Uncoupling proteins in mitochondrial processes. For example, uncoupling proteins are known to prevent exaggerated ROS generation. Additionally, they are involved in the transfer of mitochondrial substrates, mitochondrial calcium uniport and the regulation of thermogenesis (reviewed in [76]). Wu et al. reviewed the association between these findings and AD. They pointed out that no publications investigating a direct link between Uncoupling proteins and AD were available in 2010. Since then only a few more examinations regarding this subject have been published. In 2012 Scheffler et al. published data showing that ATP levels were increased in APP-transgenic mice with Uncoupling protein 2 knockout (Ucp2ko), while AD pathology was improved [77]. *In vitro* data of Jun et al. are contrary with these findings as they suggest that Ucp2 protects primary neurons against Aβ toxicity [78]. Moreover, Montesanto et al. showed that a genetic variant in human Ucp4 affects the predisposition to LOAD and alters the effect of APOE-ε4 on AD risk [79]. Our current knowledge is insufficient to describe the effect of Uncoupling proteins on AD pathology, but it proves that an association exists which should be further analysed.

Another mitochondria-related target for AD is Aβ-binding alcohol dehydrogenase (ABAD). This enzyme is upregulated in neurons affected during AD. A consequence of an interaction between Aβ and ABAD is altered mitochondrial function [80]. Additionally, Rhinn et al. showed that an inhibition of this binding causes a reduced Aβ accumulation and improved mitochondrial function [81]. Furthermore, Aβ can directly interfere with the production of NO and ATP [67,82].

As mentioned before, mutations in mtDNA play an important role in the development of AD. Analyses of isolated mtDNA from blood and brain tissue of AD patients display conflicting results [83,84]. Therefore, the hypothesized connection between mutations in mtDNA and the accumulation of Aβ is still controversially discussed [85,86]. Some researchers demonstrated an association between mtDNA and AD with cytoplasmic hybrid cells (cybrids). After a depletion of original mtDNA the same cells are repopulated with mtDNA from AD patients to generate cybrids. These cybrids had a lower mitochondrial membrane potential and secreted doubled amount of Aβ40 and Aβ42. Furthermore, this experiment showed that cells with mitochondrial DNA from AD patients have a higher affinity to activate apoptotic pathways [87,88]. In addition, Scheffler et al. characterized several APP-transgenic mouse strains with mutations in mtDNA and revealed reduced plaque burden and lower levels of soluble Aβ accompanied by upregulated ATP levels [77].

Altogether, mutations in mtDNA and oxidative stress are related to alterations in several mitochondrial processes, that might contribute to the development and progression of AD.

1.5. Mouse models – a highly adaptive tool for investigation of factors contributing to AD

Mouse models are a good tool to investigate mechanisms causing or contributing to diseases like AD. An advantage of mouse models is that numerous genetic modifications can be implemented to characterize their effects on AD. In the resulting mouse model consequences of genetic alteration can be examined under *in vivo* conditions. Therefore, results are as close to human conditions as possible without performing investigations in humans.

Mice are model organisms that have been studied extensively. Within this study, they help to investigate AD in an *in vivo* model. Before starting an experiment with mice, it is important to

choose an appropriate mouse model. This is a difficult decision which will either support or undermine the reliability of results. Several aspects should be considered when choosing a suitable mouse model.

First, the choice of background strain must fulfil some requirements. Usually scientists need a mouse model with specific genetic modifications that are available in a specific background. Still, all investigated mouse strains must have the same genetic background to guarantee a comparability of results. If this condition is not met, mice must be crossed to the same background. Additionally, it should be considered which background is the best choice to mimic the disease model of interest. Even by taking all this in consideration it is still difficult or even impossible to make a correct decision as background strains are still not fully characterized.

In AD research a broad range of mouse models expressing various AD-specific characteristics is available. Some mouse strains express variants of APP-transgene which lead to the formation of plaques at various times and with varying speed i.e. APP/PS1, APPSwDI, Tg2576 [89-91]. Other mouse strains display both plaque and tangle pathology. Although Oddo et al. generated a mouse model coexpressing APP KM670/671NL, MAPT P301L and PSEN1 M146V, its reliability was recently doubted [92].

My investigation includes the characterizations of mice with ABC transporter knockouts, mutations in mtDNA and a knockout of Uncoupling protein 2 in an AD mouse model. The AD mouse model which was chosen for this investigation is APP/PS1. Its advantage is that first plaques already occur at the age of 45 days and that it exists in the most common background strains, B6 and FVB. Therefore, this AD mouse model offers the opportunity to describe the influence of several factors within short time and it has the flexibility to be compared to genetically modified mouse strains in C57BL/6J (B6) and FVB/N (FVB) background. While ABC transporter knockout strains are commercially available solely in FVB background, most mouse strains with mitochondrial alterations have a B6 background. Crossings of strains to change the genetic background requires at least ten generations. Hence, they are highly time-consuming.

2. Aim

Since the first description of AD in 1906 several studies have revealed numerous factors like nutrition, drugs, genetics, mitochondrial and ABC transporter function contributing to the development of AD. Most studies have investigated these factors solely without considering the interaction with other factors. Therefore, the aim of this study is to examine interactions between some factors which are known to affect AD pathology.

As AD is a highly age-related disease, the first step is a profound analysis in non-transgenic mice to evaluate how ABC transporters, neuroinflammation, nuclear and mitochondrial DNA are associated to aging. Afterwards their effects on AD pathology are investigated in APP-transgenic mice.

Questions to be answered are:

1. How do background strains B6 and FVB differ and how do they affect AD pathology?
2. What are the consequences of mtDNA mutations and Ucp2 knockout and how do they interfere with AD pathology?
3. Do changes in nuclear or mitochondrial DNA have a stronger influence?
4. What effects on AD do ABC transporter knockouts have in B6 and FVB background?
5. How are ABC transporter knockouts, nuclear and mitochondrial DNA associated to neuroinflammatory responses in non- and APP-transgenic mice?

Taken together, results will help to discuss the hypothesis that AD is a disease triggered by a complex interaction of factors like neuroinflammation, alterations of nuclear and mitochondrial DNA as well as mitochondrial and ABC transporter dysfunction.

3. Materials and Methods

3.1. Materials

3.1.1. Chemicals

100 bp plus DNA ladder	Bioron GmbH, Germany	304105
Bond™ Dewax Solution	Leica Biosystems Nussloch GmbH, Germany	AR9222
Bond™ Primary Antibody Diluent	Leica Biosystems Nussloch GmbH, Germany	AR9352
Bond™ Wash Solution 10X Concentrate	Leica Biosystems Nussloch GmbH, Germany	AR9590
Disodium phosphate (Na2HPO4)	Sigma-Aldrich Co. LLC., USA	255793-50G
Ethanol 96%	Carl Roth GmbH & Co. KG, Germany	P075.1
Ethidium bromide	Carl Roth GmbH & Co. KG, Germany	2218.1
Guanidine hydrochloride	Carl Roth GmbH & Co. KG, Germany	0035.1
Formaldehyde solution 37%	Carl Roth GmbH & Co. KG, Germany	7398.1
Paraformaldehyde	Carl Roth GmbH & Co. KG, Germany	0335.4
Pertex® Mounting Medium	Leica Biosystems Nussloch GmbH, Germany	3808706E
PhosSTOP	Roche Diagnostics GmbH, Germany	4906837001
Potassium dihydrogen phosphate (KH_2PO_4)	Merck KGaA, Germany	3904.2
Protease inhibitors (Complete-mini)	Roche Diagnostics GmbH, Germany	11836153001
Proteinase K solution	AppliChem GmbH, Germany	A4392
RNAlater®	Sigma-Aldrich Co. LLC., USA	R0901
Sodium carbonate (Na_2CO_3)	Carl Roth GmbH & Co. KG, Germany	A135.2
Sodium chloride (NaCl)	Carl Roth GmbH & Co. KG, Germany	3957.1
Tris/HCl	Carl Roth GmbH & Co. KG, Germany	9090.2
Triton X-100	Carl Roth GmbH & Co. KG, Germany	3051.3

3.1.2. Buffers and Solutions

Phosphate buffered saline (PBS)

137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH 7.4

4% buffered paraformaldehyde solution

4% (w/v) paraformaldehyde, 137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH 6.9

1X PBS Lysis Buffer

1XPBS, 1% Triton/X/100, 1 pill phosphatase inhibitor, 1 pill protease inhibitor

5M Guanidine Buffer

5M guanidine/HCl, 50mM Tris/HCl, add ddH$_2$O to 50ml

8M Guanidine Buffer

8.2 M guanidine/HCl, 82mM Tris/HCl, add ddH$_2$O to 50ml

3.1.3. Kits

Pierce™ BCA Protein Assay	Thermo Fisher Scientific Inc., USA	23225
Bond Enzyme Pretreatment Kit	Leica Biosystems Nussloch GmbH, Germany	AR9551
BondTM Polymer Refine Detection	Leica Biosystems Nussloch GmbH, Germany	DS9800
OmniMap DAB anti-rabbit	Ventana Medical Systems, Inc.	760-149
Proinflammatory Panel 1 (mouse) Kit	Meso Scale Diagnostics, LLC., USA	K15048D
VPLEX Aβ40 Peptide (4G8)	Meso Scale Diagnostics, LLC., USA	K150SJE
VPLEX Aβ42 Peptide (4G8)	Meso Scale Diagnostics, LLC., USA	K150SLE
VPLEX Aβ Peptide Panel 1 (6E10)	Meso Scale Diagnostics, LLC., USA	K15200G

3.1.4. Antibodies

Anti-GFAP, polyclonal	Dako Deutschland GmbH, Germany	Z033401
Anti-IBA1, polyclonal	Wako Chemicals, Germany	019-19741
Anti-β-Amyloid, 6F3D	Dako Deutschland GmbH, Germany	M0872

3.1.5. Equipment

Analytical scale	Sartorius AG, Germany
BenchMark ULTRA	Ventana Medical Systems
Bond-Max Autostainer	Leica Microsystems GmbH/Menarini
Dewar vessel	Karlsruher Glastechnisches Werk - Schieder GmbH
Eppendorf Research® pipettes	Eppendorf AG, Germany
EV231 power supply	PEQLAB Biotechnologie GmbH, Germany

Freezer -20 °C	Gorenje Vertriebs GmbH, Germany
Freezer -80 °C	Kryotec-Kryosafe GmbH, Germany
Histokinette STP 120	Microm International GmbH, Germany
Leica EG 1160 embedding Center	Leica Microsystems GmbH, Germany
MESO QuickPlex SQ 120	Meso Scale Diagnostics, LLC., USA
Pannoramic MIDI	3DHISTECH Ltd., Hungary
Paradigm™ Multi-Mode Microplate reader	Molecular Devices, LLC., USA
Precellys® 2.8mm zirconium oxide beads	Saphire bioscience
Fridge	Liebherr-Hausgeräte GmbH, Germany
RM 2155 microtome	Leica Microsystems GmbH, Germany
SpeedMill PLUS Homogenisator	Analytik Jena AG, Germany
Thermoshaker	EuroClone
TProfessional thermocycler	Analytik Jena AG, Germany
Universal 320R centrifuge	Andreas Hettich GmbH & Co.KG, Germany
UVsolo TS	Analytik Jena AG, Germany
VTX-3000L Vortex	LMS Consult GmbH & Co. KG, Germany

3.1.6. Software

AxioVision 4.8.1.0	Carl Zeiss Imaging Solutions GmbH, Germany
Discovery Workbench 4.0	Mesoscale Discovery
Endnote X6	Thomson Reuters Corp., USA
GraphPad Prism 6.01	GraphPad Software Inc., USA
Pannoramic Viewer 1.15.4	3DHISTECH Ltd., Hungary

3.1.7. Mouse models

All mouse models analysed in this study have either a C57BL/6J or FVB/N background. To generate comparable mouse models with specific characteristics background strains were crossbred with various mouse models as described in the following sections. Furthermore, table 3 shows the number of animals and mouse models that were used in each experiment.

These mice were bred in the animal care facilities of the Neurodegeneration Research Laboratory (NRL) at the University of Rostock and the University of Magdeburg, Germany. Animals were housed in a 12h/12h light/dark cycle at a mean temperature of 22°C with free access to food (Sniff, Germany) and water. Breeding and all experiments were conducted according the European Union and state law of the government of Mecklenburg/Western Pomerania and Saxony-Anhalt, respectively, and were approved by the local animal ethics committee.

ABC transporter knock out mice

FVB.129P2-Abcg2tm1Ahs N7, FVB.129P2-Abcb1atm1Bor N12 and FVB.129P2-Abcc1atm1Bor N12 mice were purchased from Taconic Farms/Denmark [93-95]. In 2011 Krohn et al. published data that describe how ABC transporter knockout affects Alzheimer`s disease in mice with a FVB/N background. These mice were crossbred for at least 10 generations to generate mice with a C57BL/6J background and a knockout of ABC transporters ABCB1, ABCC1 or ABCG2.

mtDNA conplastic mice

Conplastic mice were generated with inbred strains FVB/N, AKR/J, NOD/LtJ, which were purchased from Jackson Laboratory (Bar Harbor, USA). As mtDNA is inherited maternally, a female mouse with a mtDNA variant (mtFVB, mtAKR, mtNOD) was mated with a male C57BL/6J mouse for more than 10 generations [96]. Thus, desired maternal mitochondrial DNA variants (table 1) were manifested in a C57BL/6J background that has no nuclear DNA of the mitochondria donating strain anymore. Scheffler et al. published data that characterize mice with described mtDNA variants in a C57BL/6J background as well as their influence on AD.

Position (bp)	C57BL/6J	AKR/J	FVB/N	NOD/J	Gene/RNA	Amino acid substitution
7778	G	G	**T**	G	mt-Atp8	Asp $\rightarrow$ Tyr
9348	R	G	G	**A**	mt-Cox3	Val $\rightarrow$ Ile
9461	T	**C**	**C**	**C**	mt-Nd3	None
9828	A	**AA**	**AA**	**AAA**	mtRNA^{Arg}	

table 1: Overview of mitochondrial DNA variants in mtDNA strains (adapted from Scheffler et al.[77])

Ucp2 knockout mice

Ucp2ko mice were purchased from Bruno Miroux (CNRS UPR 9078, Facultè de Mèdecine Necker-Enfants-Malades, Paris, France) and have a C57BL/6J background [97].

APP/PS1-B6 and APP/PS1-FVB mice

The genetic background of APP/PS1 mice analysed within this investigation is either C57BL/6J or FVB/N. These mouse models co-express KM670/671NL mutated amyloid precursor protein and L166P mutated presenilin 1 under control of a Thy1 promoter [98]. To generate APP-transgenic mice with mitochondrial alterations or ABC transporter knockouts, mice with altered mtDNA (mtFVB, mtAKR, mtNOD), Ucp2ko or an ABC transporter knockout (ABCB1, ABCC1, ABCG2) were crossbred

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with either APP/PS1-B6 or APP/PS1-FVB. In these crossings, a female mouse of these strains was mated with a male APP/PS1 transgenic mouse. The genetic background of both mice was the same.

Nomenclature

The complexity of breedings used within this investigation requires a simplifying nomenclature for mouse strains. Therefore, strain names used in this thesis consist of up to three abbreviations which conflate the genetic features of each mouse strain (table 2).

Abbreviation	Strain characteristic
B6	Background strain C57BL/6J
FVB	Background strain FVB/N
APP	Expression of two human transgenes: amyloid precursor protein and presenilin1
B1	Knockout of ABCB1a/b
C1	Knockout of ABCC1
G2	Knockout of ABCG2
Ucp2ko	Knockout of Uncoupling protein 2
mtFVB	Mitochondrial DNA derived from mouse strain FVB/N
mtAKR	Mitochondrial DNA derived from mouse strain AKR/J
mtNOD	Mitochondrial DNA derived from mouse strain NOD/LtJ

table 2: Nomenclature of mouse strains: abbreviations and a description of associated characteristics

Additionally, a colour code was generated to simplify the comparison of results gained with different methods (table 3). One colour was assigned to each strain with altered mitochondrial function or ABC transporter knockout. In diagrams stripes visualize the presence of the APP-transgenes. Moreover, lighter colours indicate that mouse strains have a FVB background, while darker colours are used for B6 background. Diagram backgrounds also visualize different backgrounds of mouse strains. While B6 background is associated to a grey diagram background, a white one indicates FVB background.

mouse strain	colour	age [d]	animal number for						
			electro chemiluminescent assays				immunohistochemistry		
			Aβ40	Buffer sol. Aβ42	Gua. sol. Aβ42	cytokines	6F3D	IBA1	GFAP
B6		100	6	-	-	6	-	8	7
		200	6	-	-	5	-	7	5
B6-B1		100	4	-	-	5	-	0	0
		200	5	-	-	6	-	4	4
B6-C1		100	4	-	-	4	-	5	3
		200	3	-	-	3	-	6	6
B6-G2		100	5	-	-	5	-	4	4
		200	4	-	-	4	-	4	4
APP-B6		100	-	3	3	4	5	3	2
		200	-	3	3	3	5	6	6
APP-B6-B1		100	-	4	4	5	5	5	5
		200	-	0	0	0	0	0	0
APP-B6-C1		100	-	5	5	5	7	13	11
		200	-	3	3	5	3	6	6
B6mtFVB		100	4	-	-	4	-	5	5
		200	5	-	-	5	-	10	10
B6mtAKR		100	4	-	-	5	-	8	8
		200	5	-	-	5	-	8	10
B6mtNOD		100	5	-	-	5	-	10	9
		200	4	-	-	5	-	10	7
B6Ucp2ko		100	3	-	-	2	-	6	6
		200	5	-	-	4	-	7	7
APP-B6mtFVB		100	-	4	2	4	9	8	9
		200	-	5	5	5	10	10	10
APP-B6mtAKR		100	-	4	4	4	9	10	8
		200	-	4	4	5	9	8	8
APP-B6mtNOD		100	-	2	2	3	9	9	8
		200	-	6	6	4	5	10	8
APP-B6Ucp2ko		100	-	4	4	4	8	8	12
		200	-	2	3	3	8	9	8
FVB		100	3	-	-	2	-	5	5
		200	4	-	-	5	-	5	5
FVB-B1		100	4	-	-	5	-	6	5
		200	5	-	-	5	-	4	2
FVB-C1		100	5	-	-	5	-	2	2
		200	4	-	-	3	-	7	7
FVB-G2		100	5	-	-	5	-	6	6
		200	5	-	-	5	-	5	3
APP-FVB		100	-	2	3	3	8	5	9
		200	-	2	1	2	6	3	3
APP-FVB-B1		100	-	3	5	3	5	5	5
		200	-	3	3	3	5	5	5
APP-FVB-C1		100	-	4	4	5	5	8	8
		200	-	3	4	4	6	6	5
APP-FVB-G2		100	-	3	3	3	0	7	8
		200	-	3	4	2	4	6	6

table 3: Overview of groups investigated within this study

Each mouse strain is associated to a colour-pattern combination which is used in each diagram. Furthermore, this table lists the analysed animal numbers of each mouse strain per method. Mouse

strain names consist of abbreviations defining the strains characteristics. Abbreviation "APP" marks mouse models displaying Alzheimer´s disease. Abbreviations "B6" (C57BL/6J) and "FVB" (FVB/N) define the background strain. ABC transporter knockouts are expressed by adding the transporter sub family and number. Mitochondrial chimerisms are indicated by abbreviations "mtAKR", "mtFVB" and "mtNOD". Abbreviation "Ucp2ko" points to a knockout of Uncoupling protein 2. More detailed descriptions of each characteristic are included in section 3.1.7.

Immunohistochemical analyses were performed with antibodies for microglia (ionized calcium binding adaptor molecule 1 - IBA1), astrocytes (glial fibrillary acidic protein - GFAP) and amyloid β (6F3D).

3.2. Methods

3.2.1. Genotyping

Genotyping was necessary to select mice with the required gene statuses. For genotyping several gene-specific PCRs were performed to exponentially propagate DNA fragments. In the first step of a PCR DNA is melted into single-strands by thermic denaturation. Then gene-specific primers (table 4) bind to complementary single-stranded DNA fragments. These primers are elongated by a DNA polymerase which concatenates dNTPs complementary to the template. Denaturation, annealing and elongation are repeated several times. In a final step, all remaining single-stranded DNA fragments are completed to double-strands.

Gene	Gene status (size)	Primer	Primer sequence	concentration [µM]
ABCB1		*ABCB1 com for*	GAG AAA CCA TGT CCT TCC AG	0.6
	wt (600bp)	*ABCB1 wt rc*	AAG CTG TGC ATG ATT CTG GG	0.3
	ko (540bp)	*ABCB1 ko rc*	GCC TTC TAT CGC CTT CTT GA	0.35
ABCC1		*ABCC1 com for*	AAG ACA AGG GCT TGG GAT GC	0.37
	wt (900bp)	*ABCC1 wt rc*	CCA TCT CTG AGA TCT TGC CG	0.5
	ko (600bp)	*ABCC1 ko rc*	GGA GCA AAG CTG CTA TTG GC	0.1
ABCG2		*ABCG2 com for*	CTT CTC CAT TCA TCA GCC TCG	0.28
	wt (368bp)	*ABCG2 wt rc*	CAG TCG ATG GAT CCA CTT AGG	0.28
	ko (595bp)	*ABCG2 ko rc*	GGA GCA AAG CTG CTA TTG GC	0.28
APP/PS1	transgene (246bp)	*APPPS1 for*	GAA TTC CGA CAT GAC TCA GG	0.25
		APPPS1 rev	GTT CTG CTG CAT CTT GGA CA	0.25
Actin control		*Actin for*	CCT CAT GAA GAT CCT GAC CG	0.20
	(400bp)	*Actin rev*	GCA CTG TGT TGG CAT AGA GG	0.20

table 4: List and description of utilized primers

Samples for genotyping were obtained by tail biopsy from 20-day old mice. These tails were incubated in mouse tail lysis buffer at 55°C overnight. Afterwards, lysed tails were heated to 95°C for 30 minutes. Then, sample lysates were stored at 4°C until use.

A PCR mix for one sample was composed of 2xMaster Mix, gene-specific primers (table 4), 1µl sample lysate and pure water added to a final volume of 12µl (table 5). DNA fragments generated by primers had different sizes. Furthermore, actin primers were added to all APP-transgene testing PCRs as positive controls of the PCR reaction in each sample.

	APP		ABCB1		ABCC1		ABCG2	
H$_2$O [µl]	4.50		4.2		4.72		5.14	
Master mix [µl]	5.50		5.50		5.50		5.50	
Primer 1 [µl]	*APPPS1 for*	0.28	*ABCB1 com for*	0.60	*ABCC1 com for*	0.16	*ABCG2 com for*	0.12
Primer 2 [µl]	*APPPS1 rev*	0.28	*ABCB1 wt rc*	0.30	*ABCC1 wt rc*	0.22	*ABCG2 wt rc*	0.12
Primer 3 [µl]	*Actin for*	0.22	*ABCB1 ko rc*	0.40	*ABCC2 ko rc*	0.04	*ABCG2 ko rc*	0.12
Primer 4 [µl]	*Actin rev*	0.22	-		-		-	
Sample [µl]	1		1		1		1	
SUM [µl]	**12**		**12**		**12**		**12**	

table 5: PCR mixes for transgenes APP, ABCB1, ABCC1 and ABCG2

To determine gene status of APP/PS1, ABCC1 and ABCG2 the same PCR program was used. It started with an initialization (5 min, 95°C). This was followed by a DNA denaturation for 30 seconds at 95°C, 60 seconds annealing at 62°C and 90 seconds elongation at 72°C. DNA denaturation, annealing and elongation were repeated 35 times before the last single-stranded DNA fragments were finally elongated for 5 minutes at 72°C. The PCR program for ABCB1 had different durations and temperatures: DNA denaturation (30 sec, 95°C), annealing (60 sec, 58°C) and elongation (60 sec, 72°C). Afterwards, all PCR products were stored at 4°C until gel electrophoresis defined their size and gene status (table 4).

3.2.2. Tissue sampling

Mice were sacrificed by cervical dislocation at the scheduled age. Then they were perfused transcardially with 1xPBS. Afterwards cerebellum was dissected, and hemispheres were divided. One hemisphere was stored in 4% PFA and used for immunohistochemistry. The other one was temporarily stored in liquid nitrogen before it finally was retained in a freezer at -80°C until the start of protein analyses. As a rule, the brain was taken out immediately after cervical dislocation and perfusion. In contrast, brains for investigating the stability of cytokines after death were removed after 30 minutes, 1, 2, 3, 4, 5 or 6 hours and mice were not perfused.

3.2.3. Immunohistochemistry

Sample preparation

As mentioned in the description of tissue sampling one hemisphere used for immunohistochemistry was stored in 4% paraformaldehyde (PFA) for three to five days. Afterwards it was put in 1xPBS supplemented with 0.01% Sodium azide until dehydration and embedding in paraffin (table 6).

Step		Reagent		Incubation time (min)
1		Formalin 4% buffered		5
2		Ethanol	70%	180
3			80%	60
4				120
5	Dehydration		90%	60
6				60
7			abs.	120
8				120
9		Xylene		120
10				120
11		Paraffin 60°C		240
12				240
13		Embedding		

table 6: Protocol for embedding in paraffin

Embedded samples were cut into 4μm thick coronal sections using a microtome (Leica Biosystems). Afterwards, paraffin was removed from sections of Aβ stainings manually (table 7). In contrast, the deparaffinization of sections for IBA1 and GFAP stainings was performed by the BenchMark ULTRA (Ventana) staining automate using the same incubation steps.

Step	Reagent		Incubation time (min)
1	Xylene		5
2			5
3	Ethanol	abs.	5
4			5
5		70%	5
6			5
7	ddH$_2$O		5
8			5
9	98% formic acid		5
10	ddH$_2$O		5

table 7: Deparaffinization protocol and pre-treatment for Aβ staining

The next step was the staining of samples with protein-specific antibodies with an automated tissue stainer. While Bond-III (Menarini/Leica) was used for Aβ stainings, BenchMark ULTRA was used for IBA1 and GFAP stainings (table 8). Each antibody incubation was performed according to specific protocols described in the following sections.

Staining	Antibody	Company	Dilution	Detection kit	Immunostaining system
Aß	Aß clone 6F3D	Dako	1:200	BondTM Polymer Refine Detection kit	Bond-III
IBA1	Anti-Iba1	Wako	1:2000	OmniMap DAB anti-rabbit	BenchMark ULTRA
GFAP	Anti-GFAP	Dako	1:1000	OmniMap DAB anti-rabbit	BenchMark ULTRA

table 8: Overview of material used for Aβ, GFAP and IBA1 stainings

After antibody incubation slices were stained using immunoperoxidase detection kits according to table *7*8 followed by covering the slides (table 9). Finally, all stained tissue sections were digitized using MiraxDesk scanner at a resolution of 230nm.

Step	Reagent		Incubation time (min)
1	ddH$_2$O		5
2	Ethanol	70%	5
3			5
4		abs.	5
5			5
6	Xylene		5
7			5

table 9: Protocol for covering the slides after stainings

Aβ staining

Stainings of Aβ plaques were performed with a BondTM Polymer Refine Detection kit following a staining protocol established in the Pahnke lab. For this staining 6F3D was used as a primary antibody. After deparaffinization slices were blocked with peroxide for 5 minutes. Then they were washed with Bond Wash solution three times before and after the incubation with primary antibody 6F3D for 15 minutes. This step was followed by an incubation step with Post Primary solution for 8 minutes before further three washing steps with Bond Wash solution.

The next steps are for implementation of detection and counter staining. Detection was realized by incubations with polymer reagent for 8 minutes and with mixed DAB Refine for 10 minutes. Between these steps slides were washed three times (2x with Bond Wash solution, 1x with deionized water).

Before counter staining slides were washed three times with deionized water. Then they were incubated with haematoxylin for 5 minutes. The final step was another washing step with deionized water, Bond Wash solution and again deionized water.

IBA1 and GFAP staining

Stainings of microglia (IBA1) and astrocytes (GFAP) were performed with BenchMark ULTRA (Figure 2). The first step was a deparaffinization. Overall, protocols for both stainings are similar. Pre-treatment and primary antibodies were different. While an IBA1 staining was pre-treated with a pre-diluted cell conditioning solution (UltraCC1), GFAP staining was pre-treated with protease (Protease 1).

Afterwards sections were incubated with the primary antibody that was included in the utilized kit for 16 minutes. The applied detection kit OmniMap DAB anti-rabbit contains multimer molecules that are conjugated to horseradish peroxidase (HRP) and bind to primary antibodies. After this amplification step sections were treated with DABMap kit. This included an incubation with DAB chromogen for 12 minutes. During this incubation, DAB chromogen reacts with HRP and H_2O_2 to generate an amplified brown staining. The final step was a counter staining which consisted of an incubation with haematoxylin II for 4 minutes and a post counterstaining with Bluing reagent for 4 minutes.
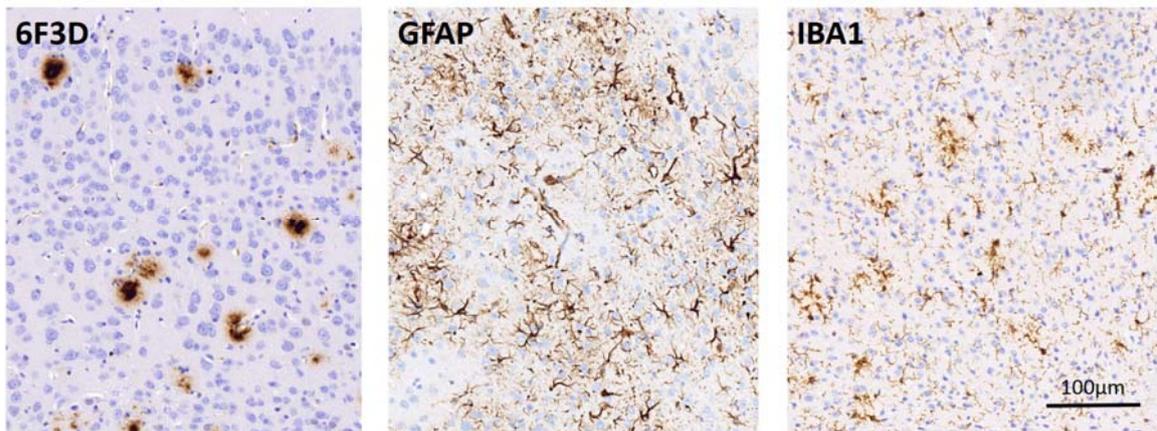
Figure 2: Exemplary stainings of Aβ plaques (6F3D), astrocytes (GFAP) and microglia (IBA1)

Immunohistochemistry analysis

Analyses of immunohistochemistry were performed with an AxioVision software package. AxioVision software was used to program macros for semi-automatic analysis of stainings. The macros for analysing stainings with IBA1, GFAP and 6F3D are based on a macro described by *Scheffler et al*. [99]. First, cortical regions of interest were defined on the scans. Afterwards, the analysed cell type was segregated due to its DAB-staining which is recognized in the green channel. Digital processes were performed based on resulting binary pictures and quantified brain area covered with the investigated cell type. Finally, this cell type area was normalized to $10mm^2$ to compare differences between mouse models. In the following steps of Aβ plaque analyses, plaques were categorized as small ($<400\mu m^2$), medium ($400\text{-}700\mu m^2$), and large ($>700\mu m^2$) plaques.

3.2.4. Protein biochemistry

Sample preparation

The preparation of samples for protein measurements was performed according to a recommendation by Mesoscale Discovery, the provider of kits for Aβ40, Aβ42 and cytokine measurements. Due to sample limitations, all measurements were based on the same sample preparation, although the mentioned recommendation refers exclusively to cytokine measurements of mouse brain tissue with assay kit "Proinflammatory Panel 1 VPlex" supplied by Mesoscale Discovery.

As mentioned before hemispheres for protein analyses were stored at -80°C. Immediately after taking samples out of the freezer 200µl 1xPBS lysis buffer per 100mg brain were added. Samples were thawed for at least 30 minutes on ice. Next, samples were homogenized with the SpeedMill using pre-programmed protocol "Tissue Soft 2". Then samples were centrifuged at 4°C with 14.000rpm for 15 minutes. The resulting supernatant was transferred into another tube.

Supernatants as well as pellets were stored at -80°C until measurements. Cytokine levels and the amount of Aβ were measured in the supernatant.

Most measurements of Aβ40 as well as buffer-soluble and guanidine-soluble Aβ42 were performed for the characterization of mouse strains. For this purpose, supernatants as well as pellets were further processed. A mixture of 50µl supernatant and 30.5µl guanidine buffer 2 was well vortexed, centrifuged (14.000rpm, 4°C) and diluted according to their APP-transgene status and age (table 10). Dilution in H_2O was performed gradually and diluent 35 was added only in the last dilution step due to the limited volume of diluent 35 that is provided with the kits from Mesoscale Discovery.

Samples measured for the comparison of Mesoscale Discovery kits with two different antibodies (4G8 and 6F3D) were processed according to a different protocol. It is the same protocol that was applied in previous experiments by Krohn et al. and Scheffler et al. After hemispheres stored at -80°C were removed from the freezer, they were homogenized with the SpeedMill using pre-programmed protocol "Tissue Soft 1". Afterwards Carbonate buffer was added to 20mg homogenate. Then this mixture was homogenized once more with the same program followed by a centrifugation (14.000rpm, 4°C), 20 min). 8M guanidine buffer was added to the resulting supernatant. After this mixture has been vortexed, it was stored at -20°C until the measurement. 5M guanidine buffer was added to the pellet. Then this mixture was shaken for three hours at 1.500 rpm and stored at -20°C until the measurement.

Total protein quantification

Depending on the composition of the used lysis buffer, the total protein concentration of samples was quantified with a BCA^{TM} Protein Assay or by spectroscopic analysis at 280nm. The total protein amount of all samples containing Triton X-100 was quantified with Pierce^{TM} BCA Protein Assay Kit (ThermoFisher Scientific). The assay was performed according to manufacturer's instruction. Therefore, samples were thawed for at least 30 minutes, diluted as mentioned in table 10 and intensively vortexed. Bovine serum albumin (BSA) was used as reference protein. Afterwards, the protein amount was measured using the SpectraMax Paradigm^{TM} Multi-Mode Microplate reader at a wavelength of 562nm.

Group	age [d]	Sample type	Dilution factor
Non-transgenic	100	supernatant	33
	200		
APP-transgenic	100	supernatant	33
		pellet	5
	200	supernatant	33
		pellet	5

table 10: Dilution factors for protein quantification

MSD Sandwich Immunoassays

MSD Sandwich Immunoassays quantify the concentration of specific proteins or molecules in complex biological samples by measuring the emission of light signals. The stimulation of electro chemiluminescent labels generates these light signals with electricity in a suitable chemical environment. These Immunoassays are performed on a microtiter plate with 96 wells and up to 10 spots per well. Due to multiple spots per well it was possible to quantify several analytes at the same time from a single, small-volume sample.

Furthermore, these immunoassays are sandwich assays (Figure 3). Specific capture antibodies are coated to working electrodes on the bottom of MSD microplates. Electro chemiluminescent labels (SULFO-TAGs) conjugated to detection antibodies bind the analytes after their transfer into the wells.
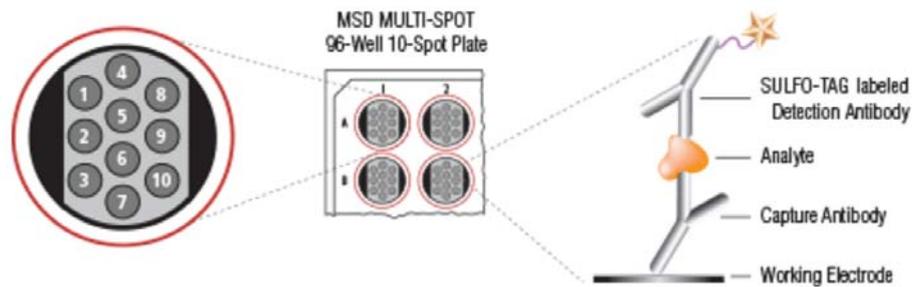
Figure 3: Visualization of sandwich assay and the placement of analyte capture antibodies on multiplex plate (adapted from MesoScale Discovery)

Quantification of cytokine levels

The levels of a defined cytokine selection were measured with the Proinflammatory Panel 1 kit supplied by Mesoscale Discovery. Supernatants of samples were thawed for at least 30 minutes on ice and thoroughly vortexed before being diluted 1:5.

Plates from the mentioned kit were incubated with 50µl diluted sample per well (RT, 2h). For each incubation step of this protocol the adhesive film sealed plate was shaken at 200rpm. The incubation with samples was followed by three washing steps with 150µl 1xPBS per well. Afterwards each well was incubated with 25µl 1x detection antibody solution for two hours. Then another washing step was followed by the addition of 150µl Read Buffer per well. Finally, the plate was read with a MESO QuickPlex SQ 120. The Discovery Workbench 4.0 software was used to analyse the measured values.

Quantification of buffer-and guanidine-soluble Aβ40/42 levels

The supernatants and pellets of non- and APP-transgenic mice prepared for Aβ measurements were kept on ice for 30 minutes and diluted according to their APP-transgene status and age (table 11). Dilution in water was performed gradually and diluent 35 was added only in the last dilution step due to the limited volume of diluent 35 that is provided with the kits from Mesoscale Discovery.

		age [d]	sample type	dilution factor
Non-transgenic	**Aβ40**	100	supernatant	2
		200	supernatant	2
APP-transgenic	**Aβ42**	100	supernatant	1640
		100	pellet	1000
		200	supernatant	3280
		200	pellet	4000

table 11: Dilution factors for Aβ40/42 quantification

In the following, supernatants and pellets of non- and APP-transgenic mice were analysed to quantify Aβ40 and Aβ42 levels with three different kits from Mesoscale Discovery: "V-PLEX Aβ40 Peptide Kit", "V-PLEX Aβ42 Peptide Kit" as well as "V-PLEX Aβ Peptide Panel 1 Kit". These kits use

anti-β amyloid antibodies, to be precise either a 4G8 or a 6F3D clone, as peptide-specific capture antibodies.

The instructions of all kits that are utilized to measure Aβ40 and/or Aβ42 are identical. First 96-well plates were blocked by adding 150µl of diluent 35 to each well. Then, plates were sealed with adhesive film and shaken at RT for one hour. Afterwards plates were washed three times with 150µl 1xPBS per well. 25µl of diluted sample or calibrator were added to each well. This was followed by an incubation step (700rpm, RT, 2h). Subsequently, the plate was washed once again as described before. After this step, it was prepared for measurement by adding 150µl 2xRead Buffer T. Finally, the plate was read with MESO QuickPlex SQ 120 followed by analysing the results with Discovery Workbench 4.0.

3.2.5. Statistical analysis

Prism version 6 (GraphPad Software, USA) was used to perform statistical analysis. The performed statistical tests were 2way ANOVA with post hoc Holm-Sidak correction, student´s t-test and multiple t-test followed by Sidak-Bonferroni test to determine statistical significances. p values of $p \leq 0.05$ were considered statistically significant. All results were presented as mean with standard error of mean (SEM).

2way ANOVA tests with post hoc Holm-Sidak correction were performed for different subjects of this investigation, each represented by a single diagram based on a separate and independent statistical test. Furthermore, in all calculations regarding cytokine levels values below detection range were set zero after excluding methodological mistakes.

4. Results

As the aim of the study is to demonstrate how neuroinflammation, alterations of nuclear and mitochondrial DNA as well as mitochondrial and ABC transporter dysfunction interact, 23 mouse strains (46 groups) were investigated. In this section, the results of immunohistochemistry (stainings of microglia, astrocytes and Aβ plaques), quantifications of Aβ40, Aβ42 and cytokines are summarized. Within each analysis-chapter results are sorted according to the following subtopics/strain comparisons:

1. Influence of nuclear and mitochondrial DNA
2. Influence of ABC transporter knockouts in APP- and non-transgenic mice
3. Influence of genetic background on APP- and non-transgenic mice with and without ABC transporter knockouts
4. Influence of altered mitochondrial function in APP- and non-transgenic mice

Some mouse strains, especially background strains B6, FVB, APP-B6 and APP-FVB, are compared multiple times within different subtopics. Therefore, it is possible that changes within background strains or to each other are significant in statistical analysis of some subtopics but not in other ones.

4.1. Immunohistochemistry

Mouse strains were characterized by immunohistochemical analyses which include stainings of microglia, astrocytes and Aβ plaques. Furthermore, some mouse strains were compared regarding more than one subject. For that reason and to ensure a clear overview, only the statistical results of stainings are summarized in the following. Panels consisting of exemplary stainings of each strain can be found in the appendix.

4.1.1. Influence of nuclear and mitochondrial DNA in APP- and non-transgenic mice

Cortical areas of 100 days and 200 days old B6, FVB and B6mtFVB mice with and without APP-transgene were analysed to investigate whether and how nuclear and mitochondrial DNA affect immunological state (microglia and astrocyte coverage) in brains with and without plaque formation. Furthermore, the influence of different genetic backgrounds and mitochondrial DNA on plaque formation, especially plaque size, plaque number and the distribution of small, medium, and large plaques was analysed. All analyses were performed semi-automatically with AxioVision.
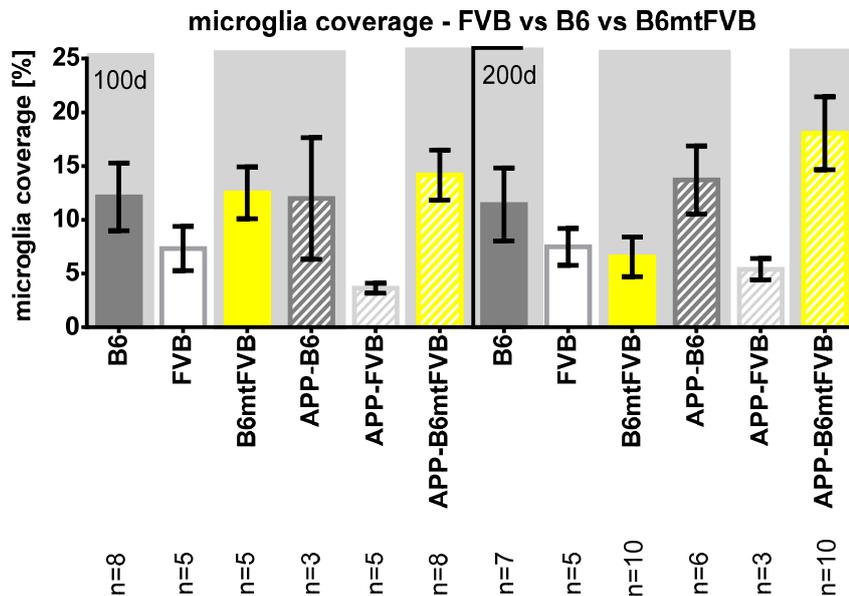
Figure 4: Neither altered nuclear DNA nor altered mitochondrial function affect microglia coverage in cortical area of non-transgenic and APP-transgenic mice.
On the left side microglia coverages of 100 days old non-transgenic and APP-transgenic B6, FVB and B6mtFVB mice are shown. The results of 200 days old mice are on the right side. Cortical areas at Bregma +1.8mm were stained with microglia-specific antibody IBA1. Column background illustrates the nuclear background of mouse strains. (grey – B6 background; white – FVB background). 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *p≤0.05

Cortices of 100 and 200 days old B6, FVB and B6mtFVB mice with or with APP-transgene were stained with IBA1 and analysed to investigate the microglia coverage in these strains (Figure 4). Comparing the microglia coverage in 100 and 200 days old B6, FVB or B6mtFVB mice with or without APP-transgene did not reveal significant differences regarding age or nuclear background.

Astrocyte coverage
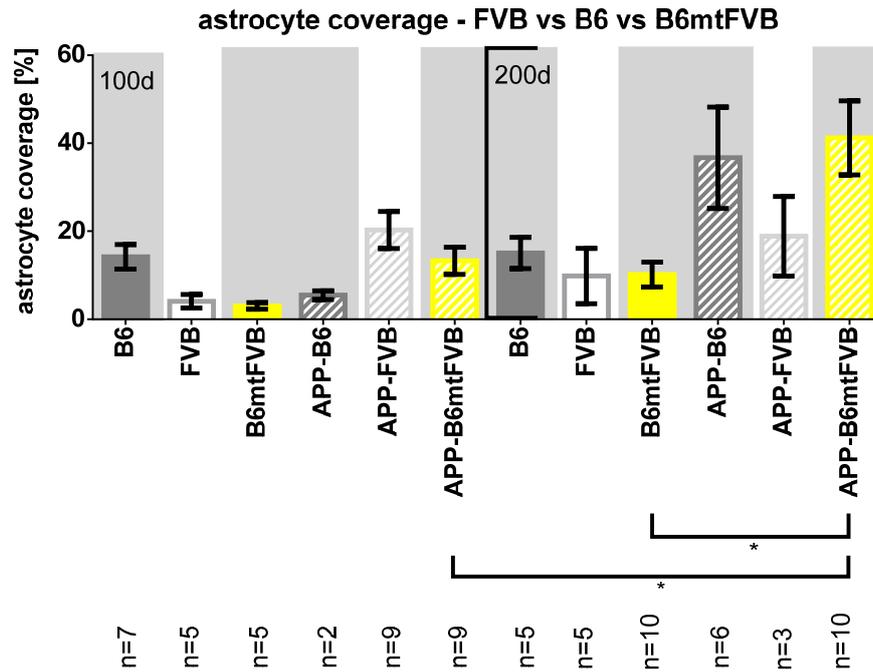
Figure 5: Neither altered nuclear background nor altered mitochondrial function affect astrocyte coverage in cortical area.
On the left side astrocyte coverages of 100 days old non-transgenic and APP-transgenic B6, FVB and B6mtFVB mice are shown. The results of 200 days old mice are shown on the right side. Cortical areas at Bregma +1.8mm were stained with astrocyte-specific antibody GFAP. Column background illustrates the nuclear background of mouse strains (grey – B6 background; white – FVB background). 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *p≤0.05

Non-transgenic and APP-transgenic B6, FVB and B6mtFVB mice were stained with anti-GFAP antibody. While a comparison of non- and APP-transgenic B6 and FVB mice (100 and 200 days old) revealed no significant differences in astrocyte coverage, 200 days old APP-B6mtFVB mice show significantly significant higher coverage than 100 days old APP-B6mtFVB and 200 days old B6mtFVB mice (Figure 5). Other comparisons to non- and APP-transgenic B6mtFVB mice are not significant.

Plaque analysis
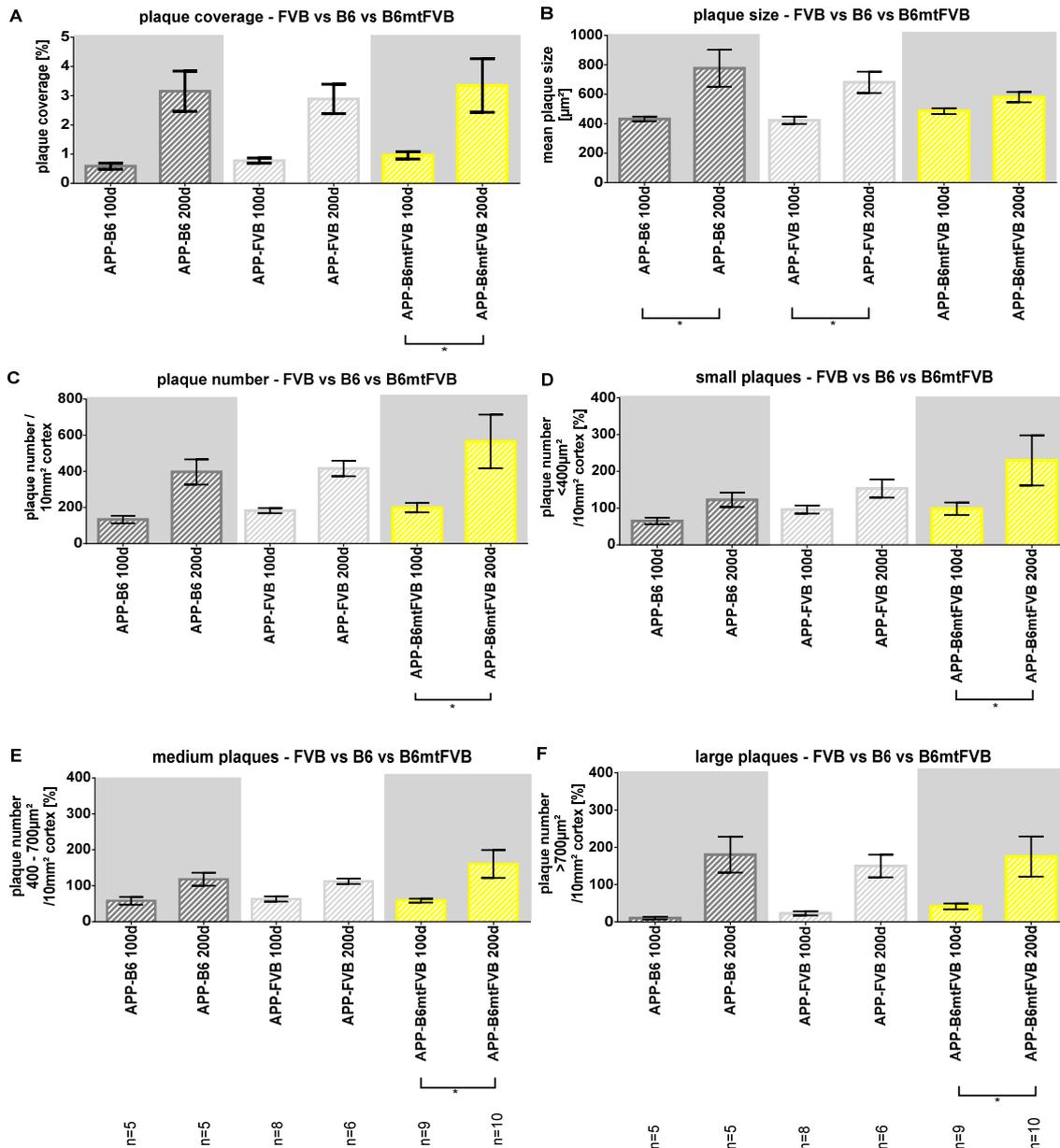
Figure 6: Plaque analyses of APP-B6, APP-FVB and APP-B6mtFVB mice
Plaque coverage (A) and the number of all, small, medium, and large plaques (C-F) rise with increasing age only in APP-B6mtFVB mice, while plaque size (B) is enhanced in 200 days old APP-B6 and APP-FVB mice compared to 100 days old equivalents. Cortical areas at Bregma +1.8mm were stained with antibody 6F3D. Column background illustrates the nuclear background of mouse strains (grey – B6 background; white – FVB background). 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *p≤0.05

Cortical areas of APP-B6, APP-FVB and APP-B6mtFVB mice were stained with 6F3D. Although no significant effects on plaque formation were recognized by comparing age-matched APP-transgenic B6, FVB and B6mtFVB mice, some age-related differences between 100 and 200 days old mice are obvious (Figure 6). All plaque characteristics increase with rising age in APP-transgenic B6, FVB and B6mtFVB mice, even if these increases are not significant.

At the age of 200 days, plaque coverage (+2.3%, A) as well as plaque number (+365 plaques/10mm^2 cortex, B) of APP-B6mtFVB mice are significantly higher compared to 100 days. Additionally, plaque number of all sizes rises from 100 to 200 days. At the age of 200 days 10mm^2 cortical area contain a higher number of small (+131), medium (+101) and large (+133) plaques than 100 days old APP-B6mtFVB mice. In contrast to APP-B6 and APP-FVB mice the mean plaque size of APP-B6mtFVB mice does not change. The plaque size of APP-B6 increases from 432µm^2 at 100 days to 777 µm^2 at 200 days, while for APP-FVB mice it rises from 423µm^2 (100 days) to 681µm^2 (200 days).

4.1.2. Influence of altered mitochondrial function in APP- and non-transgenic mice

An important aspect of this study is the analysis how aging and AD are affected by modified mitochondrial functions. Modifications in mitochondrial function are caused by variations in mitochondrial DNA (mtFVB, mtAKR, mtNOD) or by a knockout of a nuclear encoded protein (Ucp2). That is why, the cortical areas of 100 days and 200 days old B6, B6mtFVB, B6mtAKR, B6mtNOD and B6Ucp2ko mice with and without APP-transgene were analysed. The results of these analyses show the effects on immunological state (microglia and astrocyte coverage) and on plaque formation, especially plaque size, plaque number and the distribution of small, medium, and large plaques. All analyses were performed semi-automatically with AxioVision.

Microglia coverage
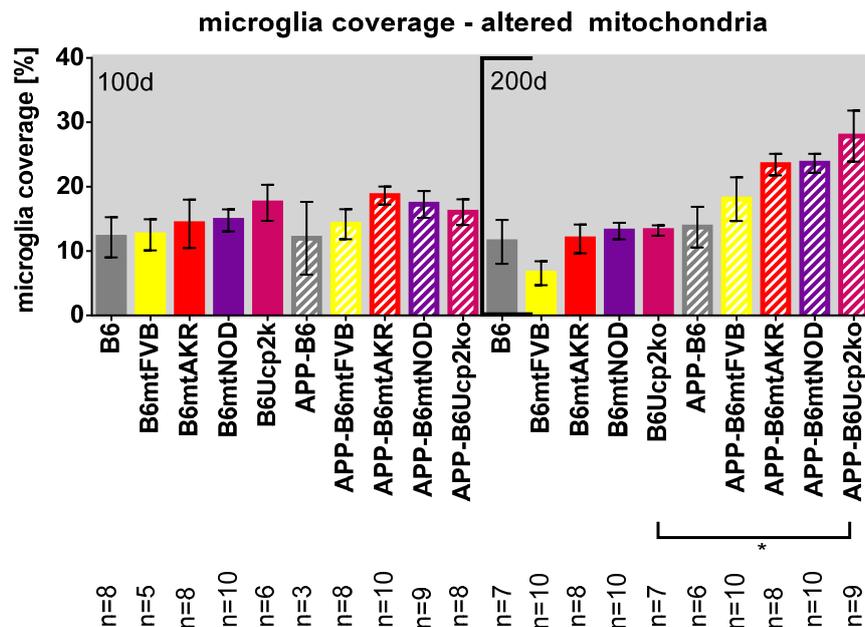
Figure 7: The microglia coverage of 200 days old APP-B6Ucp2ko mice is significantly higher than in B6Ucp2ko mice.
Microglia coverage of 100 (left) and 200 (right) days old non-transgenic and APP-transgenic mice with altered functions due to mtDNA mutations are shown. All mouse strains have a B6 background. Cortical areas at Bregma +1.8mm were stained with microglia-specific antibody IBA1. 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *p≤0.05

Cortices of mouse models with mutations in mitochondrial DNA (mtFVB, mtNOD, mtAKR) or a knockout of Uncoupling protein 2 were stained for IBA1 and analysed to determine the effects of varying mitochondrial function on microglia coverage in non-transgenic and APP-transgenic mice. All mice with altered mitochondrial function have a nuclear B6 background.

Microglia coverages of 100 days old B6 mice with altered mitochondrial functions are not significantly different, not even if non-transgenic and APP-transgenic strains are compared (Figure 7). Comparisons of 200 days old B6, B6mtFVB, B6mtAKR and B6Ucpko mice with their equivalent APP-transgenic strain revealed a significant higher microglia coverage in APP-B6-Ucp2ko compared to B6-Ucp2ko at the same age. No significant effects of altered mitochondrial function were discovered by comparing the other strains at the age of 200 days to each other and to their 100 days old equivalent strain. To sum up, solely in a Ucp2ko strain at the age of 200 days the APP-transgene causes a significant increase of microglia coverage.

Astrocyte coverage
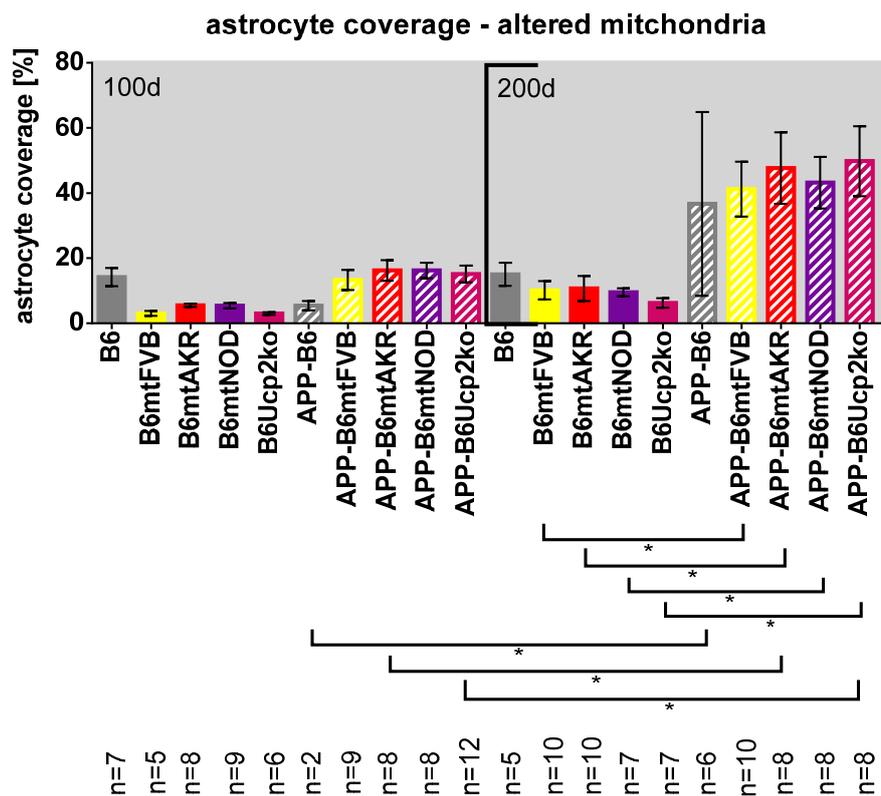
Figure 8: Effects of alterations in mitochondrial and nuclear DNA on astrocyte coverage in non- and APP-transgenic B6 mice
200 days old APP-transgenic mice with altered mitochondrial function have significantly higher levels of astrogliosis than their non-transgenic equivalents. In APP-transgenic B6mtFVB, B6mtAKR and B6-Ucp2ko mice astrocyte coverage increases astrocyte coverage between 100 and 200 days. Cortical areas at Bregma +1.8mm were stained with astrocyte-specific antibody GFAP. 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, * p≤0.05

Astrocyte coverages of non-transgenic and APP-transgenic mice with mitochondrial mutations or a knockout of Uncoupling protein 2 were examined after cortical areas of these strains have been stained with GFAP and semi-automatically analysed. All mouse strains in Figure 8 have a B6 background.

While non-transgenic and APP-transgenic B6, B6mtFVB, B6mtAKR and B6mtNOD mice have similar levels of astrocyte coverages at the age of 100 days, this fact changes at the age of 200 days (Figure 8). The APP transgene has a significant effect on all 200 days old mice with altered mitochondrial

function (B6mtFVB, B6mtNOD, B6mtAKR). This transgene increases the level of astrocyte coverage significantly. Furthermore, in APP-B6mtFVB, APP-B6mtAKR and B6Ucp2ko the cortical area covered by astrocytes increases significantly from the age of 100 to 200 days.

Plaque analysis
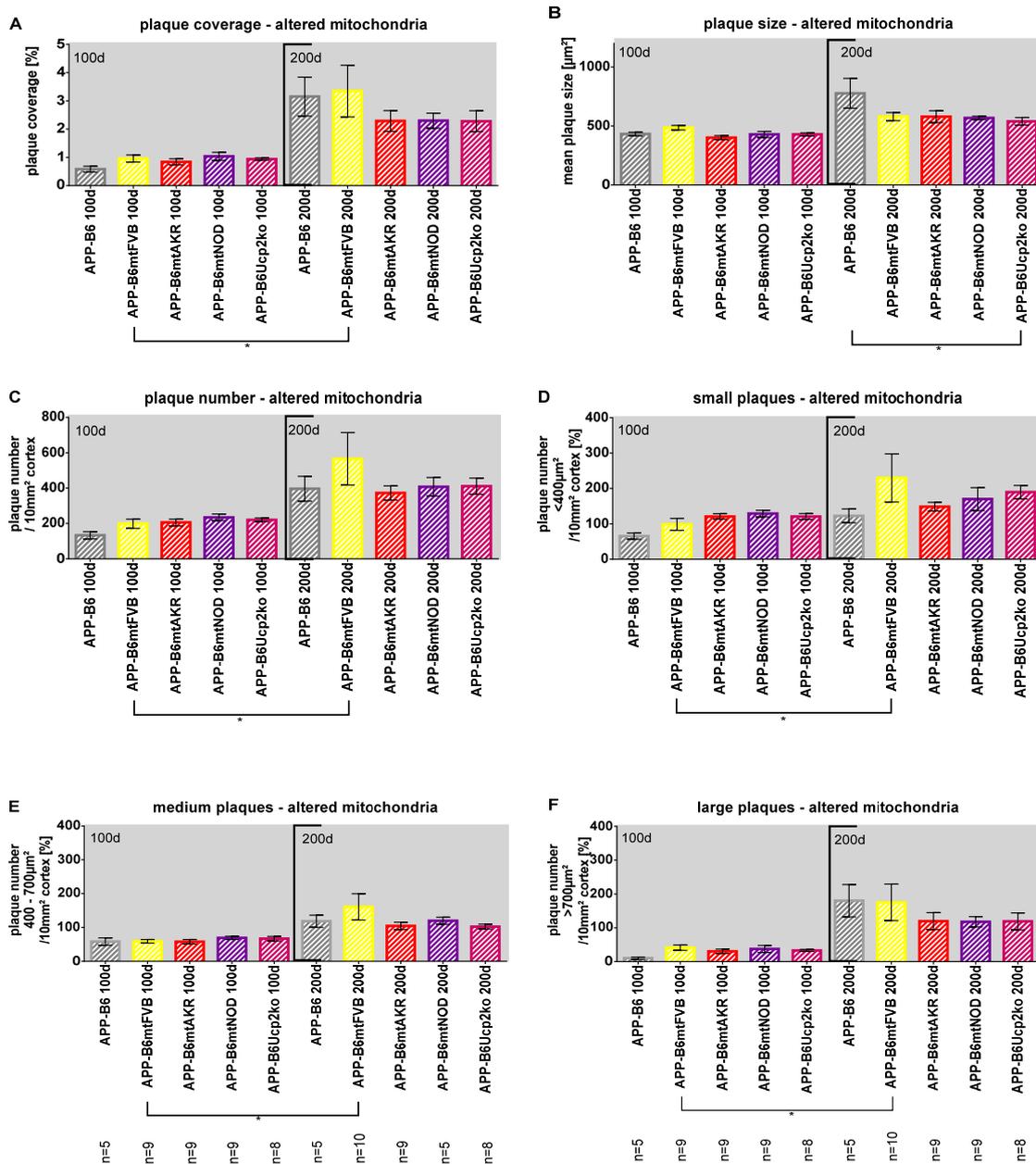
Figure 9: Effects of alterations in mitochondrial and nuclear DNA on plaque formation in non- and APP-transgenic B6 mice
The knockout of nuclear encoded protein Ucp2 in 200 days old APP-B6 mice decreases plaque size (B) significantly in comparison to age-matched APP-B6 mice. In 200 days old APP-B6mtFVB mice plaque coverage (A), plaque number (C) and the distribution of small (D), medium (E) and large (F) plaques in cortical area is enhanced in comparison to their 100 days old equivalents. Cortical areas at Bregma +1.8mm were stained with antibody 6F3D. 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, * p≤0.05

The influence of altered mitochondrial function on plaque coverage, plaque number, plaque size and the distribution of small, medium, and large plaques was investigated on 6F3D stainings in APP-transgenic mice with altered mitochondrial function. All mouse strains in Figure 9 have a B6 background.

Mouse strains with altered mitochondrial function show slight increases of plaque coverage, plaque size, the number of all, small, medium, and large plaques with rising age (Figure 9). Age-matched comparisons of these mouse strains indicate no significant differences between them except a significant decrease of plaque size in 200 days old APP-B6Ucp2ko compared to 200 days old APP-B6 mice. Additionally, comparing 100 to 200 days old mice with mutations in mtDNA and a knockout of Uncoupling protein 2 revealed no significant distinctions with one exception. In APP-B6mtFVB mice plaque coverage, plaque number and the number of small, medium, and large plaques increase significantly from 100 to 200 days.

4.1.3. Influence of ABC transporter knockouts in APP- and non-transgenic mice

The effects of ABC transporter knockouts ABCB1, ABCC1 and ABCG2 on aging and AD were analysed in genetic backgrounds B6 and FVB. In this section, the influence of ABC transporters on immunological state (microglia and astrocyte coverage) and on plaque formation, especially plaque size, plaque number and the distribution of small, medium, and large plaques are analysed and shown separately in each background at 100 and 200 days. All analyses were performed semi-automatically with AxioVision.

Microglia coverage
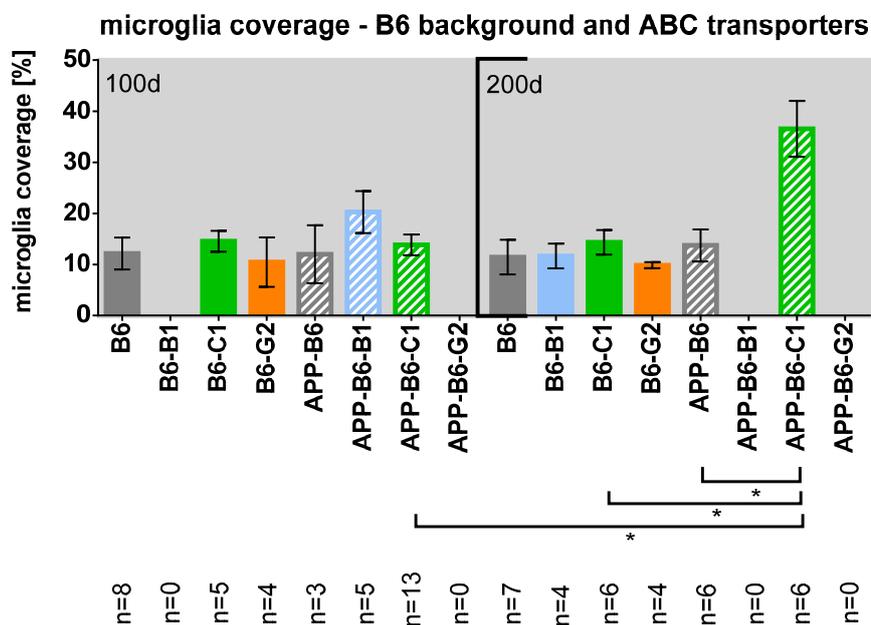
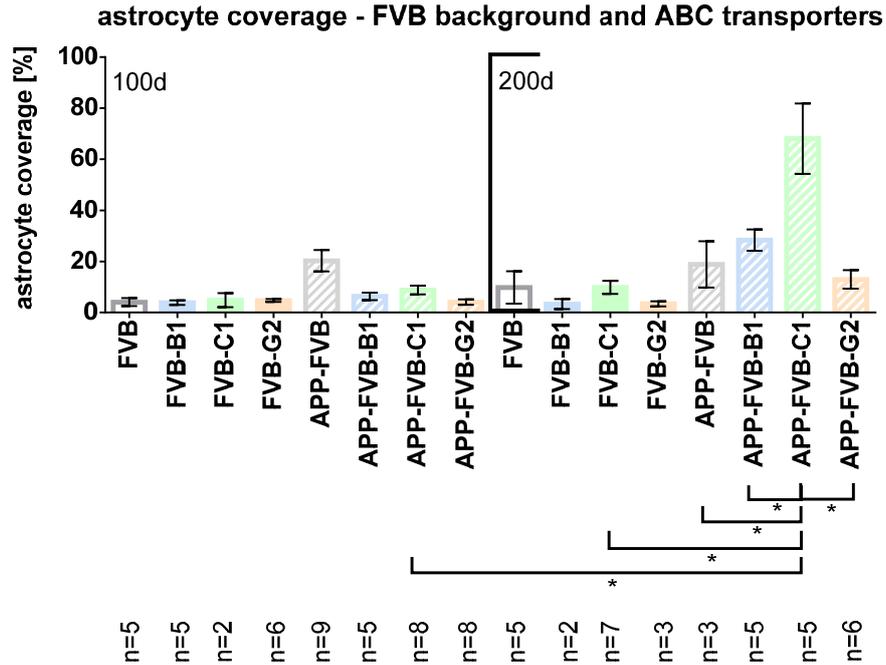**Figure 10: Effects of ABC transporters (ABCB1, ABCC1, ABCG2) on microglia coverage in cortical area of non-transgenic and APP-transgenic mice with a genetic B6 background**
The combination of age, APP-transgene and ABCC1 knockout increases microglia coverage in 200 days APP-B6 mice. Cortical areas at Bregma +1.8mm were stained with microglia-specific antibody IBA1. 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *p≤0.05

To study the influence of ABC transporters ABCB1, ABCC1 and ABCG2 on microgliosis, the cortices of 100 days and 200 days old ABC transporter knockout B6 mice with and without APP-transgene were stained with IBA1 and semi-automatically analysed. Figure 10 shows microglia coverage of ABC transporter knock out mice with a B6 background. Due to limitations of sample material, no IBA1 stainings of groups 100 days old B6-B1, 200 days old APP-B6-B1 and 100 and 200 days old APP-B6-G2 exist.

At the age of 100 days neither non-transgenic nor APP-transgenic mice with ABC transporter knockouts have significant changes in microglia coverage compared to their control (B6 or APP-B6). Comparing 100 days old ABC transporter knockout mice with each other reveals no significant differences in microglia coverage. In these non-transgenic and APP-transgenic strains 10 to 21% of cortical area are covered with microglia. At the age of 200 days APP-B6-C1 mice have significant higher microglia coverage than 200 days old APP-B6, B6-C1 and 100 days APP-B6-C1.
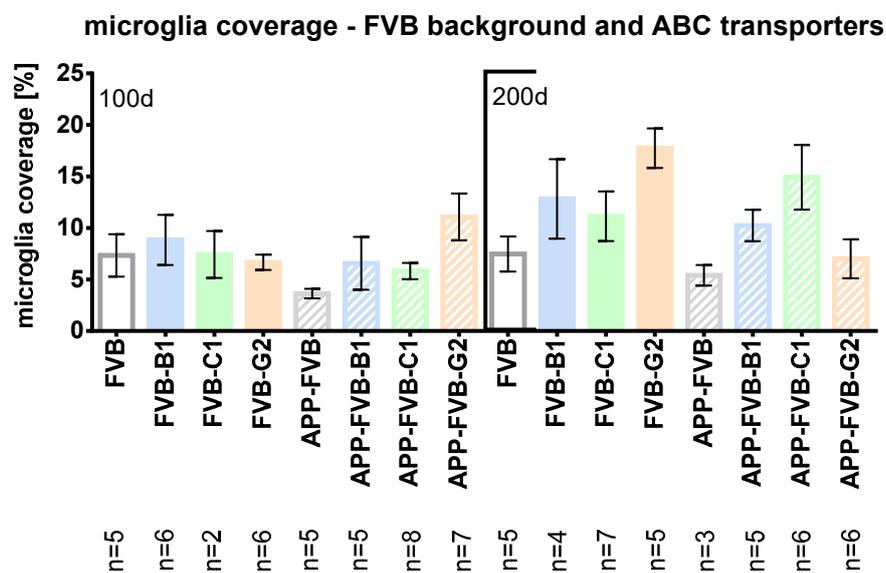
Figure 11: Effects of ABC transporters (ABCB1, ABCC1, ABCG2) on microglia coverage in cortical area of non-transgenic and APP-transgenic mice with a genetic FVB background
ABC transporters (ABCB1, ABCC1, ABCG2) do not affect microglia coverage in cortical area of mice with a genetic FVB background neither at 100 (left) nor at 200 (right) days. 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *$p \leq 0.05$

To study the influence of ABC transporters ABCB1, ABCC1 and ABCG2 on microgliosis, the cortices of 100 days and 200 days old ABC transporter knockout FVB mice with and without APP-transgene were stained with IBA1 and semi-automatically analysed. Figure 11 shows microglia coverage of ABC transporter knock out mice with a FVB background.

Overall, multiple comparisons of non-transgenic and APP-transgenic FVB mice with ABC transporter knock outs and their controls show no significant differences at the age of 100 or 200 days. Neither any ABC transporter knock out nor increasing age or the presence of an APP-transgene affect microglia coverage in mice with FVB background. Furthermore, a comparison of ABC transporter knockout strains to each other does not reveal significant differences.

Astrocyte coverage
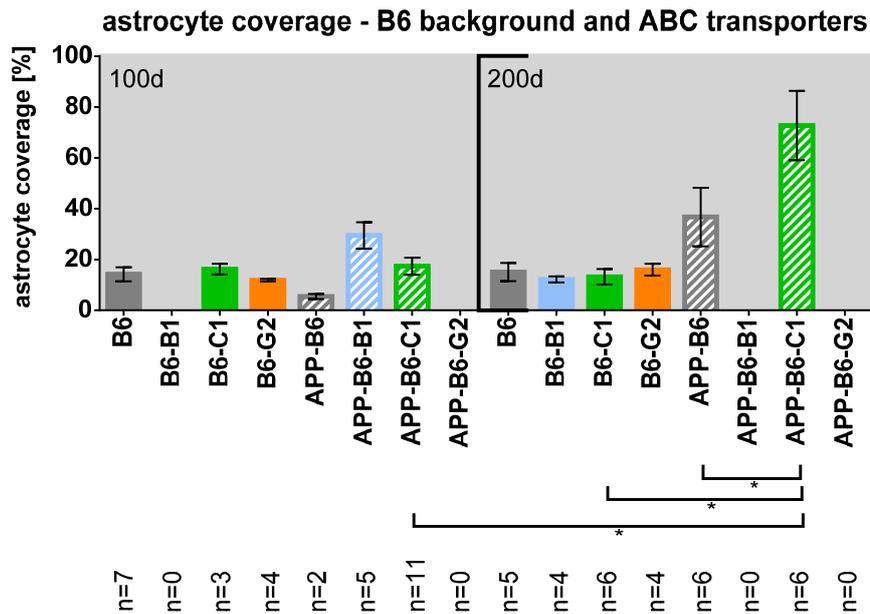
Figure 12: Effects of ABC transporters (ABCB1, ABCC1, ABCG2) on astrocyte coverage in cortical area of mice with a genetic B6 background
The combination of age, APP-transgene and ABCC1 knockout increases astrocyte coverage in 200 days APP-B6 mice. Cortical areas at Bregma +1.8mm were stained with astrocyte-specific antibody GFAP. 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, $*p \leq 0.05$

Cortices of 100 and 200 days old ABC transporter knock out mice with and without APP-transgene were stained with GFAP and semi-automatically analysed to determine the influence of ABC transporters ABCB1, ABCC1 and ABCG2 on astrocyte coverage. Figure 12 shows astrocyte coverage of ABC transporter knock out mice with a B6 background. Due to limitations of sample material, no GFAP stainings of groups 100 days B6-B1, 200 days APP-B6-B1 and 200 days APP-B6-G2 exist.

At the age of 100 days neither non-transgenic nor APP-transgenic mice with ABC transporter knockouts have significant changes in astrocyte coverage compared to their control (B6 or APP-B6). Comparing 100 days old ABC transporter knockout mice with each other reveals no significant differences in astrocyte coverage. At the age of 200 days APP-B6-C1 mice have significant higher astrocyte coverage than 200 days old APP-B6, B6-C1 and 100 days APP-B6-C1.

Figure 13: Effects of ABC transporters (ABCB1, ABCC1, ABCG2) on astrocyte coverage in cortical area of non-transgenic and APP-transgenic mice with a genetic FVB background
The astrocyte coverage of 200 days old APP-FVB-C1 mice is significantly higher compared to the other ABC transporter knock out mice, to 200 days old FVB-C1 mice and 200 days old APP-FVB mice. Furthermore, the astrocyte coverage of APP-FVB-C1 mice increased extremely from the age of 100 (left) to 200 (right) days. 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *p≤0.05

GFAP stainings of cortices from non-transgenic and APP-transgenic FVB, FVB-B1, FVB-C1 and FVB-G2 mice (100 and 200 days) were examined to find out whether ABC transporters ABCB1, ABCC1 or ABCG2 affect astrocyte coverage. Figure 13 shows astrocyte coverage of ABC transporter knock outs in FVB background.

The results indicate no significant differences in astrocyte coverage comparing control strains (FVB, APP-FVB) with their equivalent ABCB1, ABCC1 or ABCG2 knockout strain at the age of 100 days. In contrast, astrocyte coverage of 200 days old APP-FVB-C1 mice is significantly increased compared to its non-transgenic counterpart. Furthermore, cortical area of this strain is significantly more covered by astrocytes than APP-transgenic strains with no, ABCB1 or ABCG2 knockouts at the same age. Additionally, astrocyte coverage of APP-FVB-C1 mice rises significantly from 100 to 200 days.
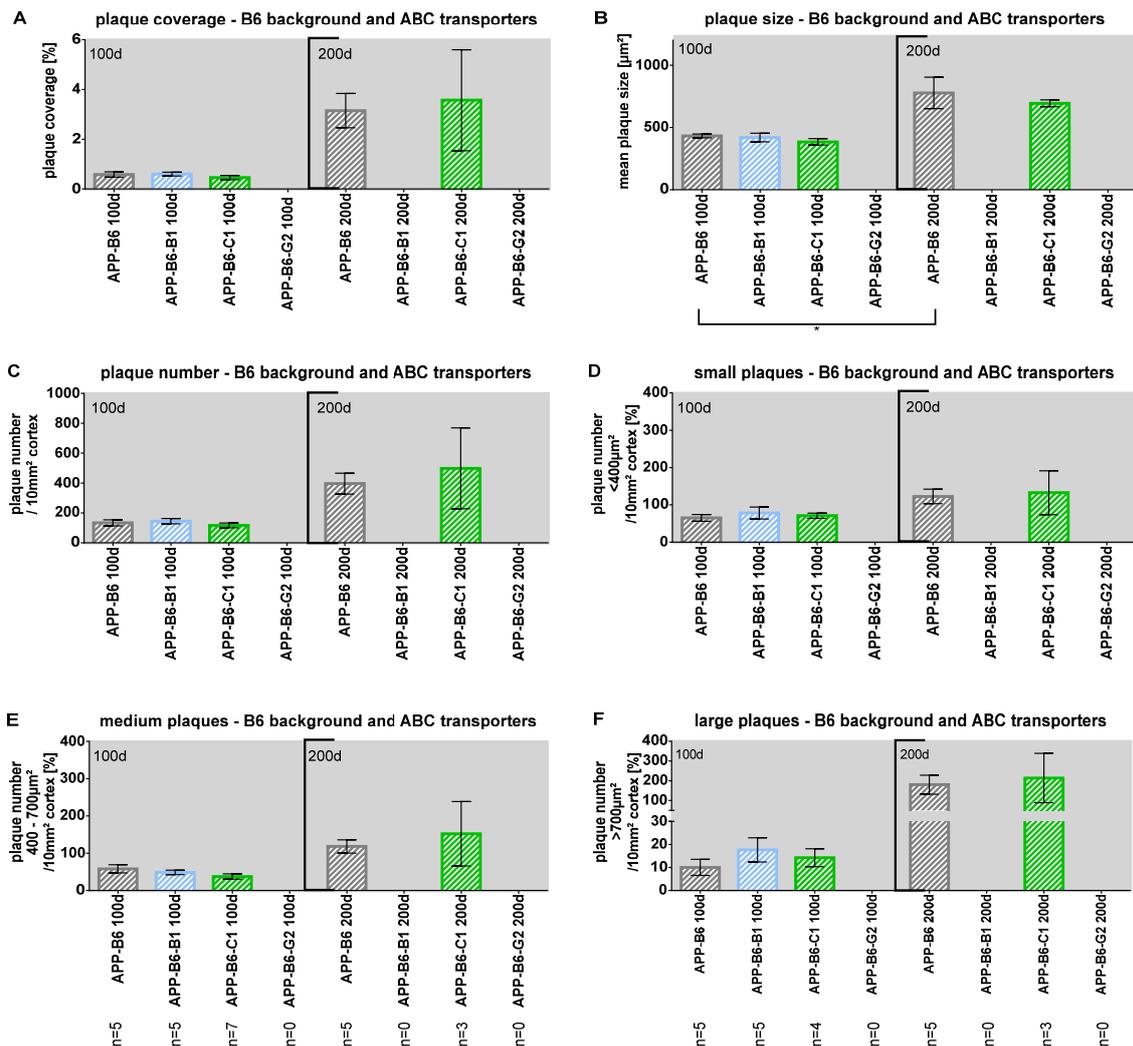
Figure 14: Effects of ABC transporter knockouts on plaque formation in 100 (left) and 200 (right) days old APP-transgenic B6 mice

ABC transporters (ABCB1, ABCC1) do not affect plaque coverage (A), plaque number (B), plaque size (C) or the distribution of small (D), medium (E) or large (F) plaques in cortical area of mice with B6 background. Cortical areas at Bregma +1.8mm were stained with antibody 6F3D. 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *$p \leq 0.05$

Cortical areas of 100 and 200 days old APP-transgenic B6, B6-B1, B6-C1 and B6-G2 mice were stained with 6F3D to check the influence of ABC transporters ABCB1, ABCC1 and ABCG2 in B6 background on plaque formation. This includes investigations on plaque coverage, plaque number, plaque size and the distribution of small, medium, and large plaques (A-F). All strains in Figure 14 have a B6 background. Due to limitations of sample material, no 6F3D stainings of the following groups exist: 100 and 200 days old APP-B6-G2 and 200 days old APP-B6-B1.

In APP-B6-C1 mice plaque coverage, plaque size, number of all, small, medium, and large plaques rise with increasing age, but this increase is not significant. ABC transporter ABCB1 does not affect plaque development at the age of 100 days. Its influence at the age of 200 days cannot be

investigated to a lack of sample material. Additionally, neither at the age of 100 days nor at 200 days ABCC1 deficiency has a significant effect on plaque formation. Comparison of ABC transporter knockout strains to each other as well as comparing them to control strain APP-B6 shows no significant changes. However, plaque size in cortices of APP-B6 mice rises significantly from the age of 100 to 200 days.
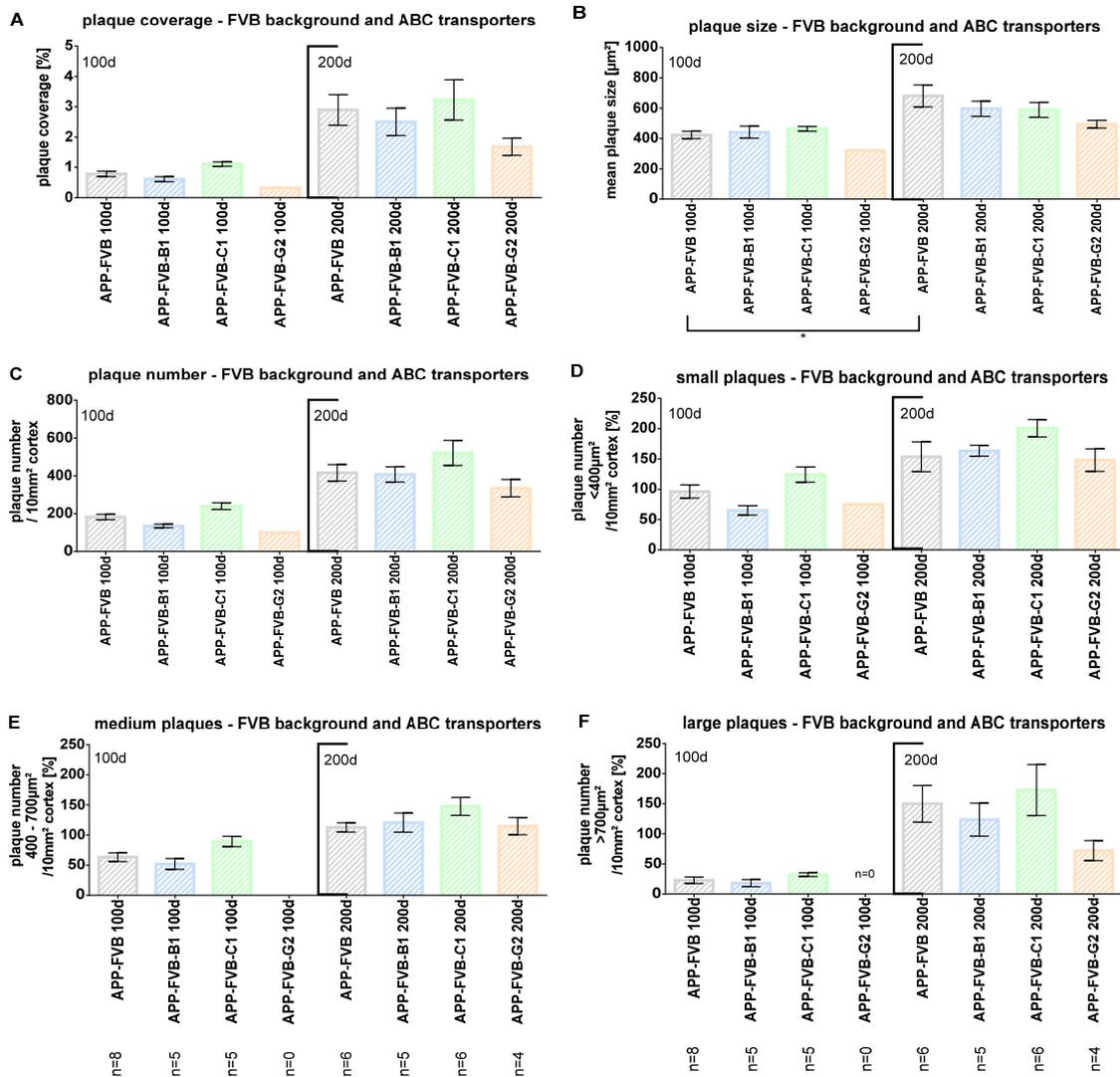
Figure 15: Effects of ABC transporter knockouts on plaque formation in 100 (left) and 200 (right) days old APP-transgenic B6 mice
ABC transporters (ABCB1, ABCC1, ABCG2) do not affect plaque coverage (A), plaque size (B), plaque number (C) and the distribution of small (D), medium (E) or large (F) plaques in cortical area of mice with FVB background. 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *p≤0.05

Plaque coverages, plaque numbers, plaque sizes and the distribution of small, medium, and large plaques of APP-FVB, APP-FVB-B1, APP-FVB-C1 and APP-FVB-G2 mice were compared between these strains by analysing 6F3D stainings of cerebral cortices. Comparisons do not indicate any significant effects of ABC transporters ABCB1, ABCC1 or ABCG2 on plaque coverage, plaque number or the distribution of small, medium, and large plaques neither at the age of 100 nor at the age of 200 days (Figure 15). Nevertheless, plaque size of 200 days old APP-FVB mice is significantly increased compared to 100 days old APP-FVB mice.

4.1.4. Influence of genetic background on ABC transporter knockouts in APP- and non-transgenic mice

To describe the influence of genetic backgrounds B6 and FVB on ABC transporters, non- and APP-transgenic ABC transporter knockout mice in B6 background are compared to their equivalents in FVB background separately at 100 and 200 days. In this section, the effects on immunological state (microglia and astrocyte coverage) and on plaque formation, especially plaque size, plaque number and the distribution of small, medium, and large plaques are shown. All analyses were performed semi-automatically with AxioVision.

Microglia coverage
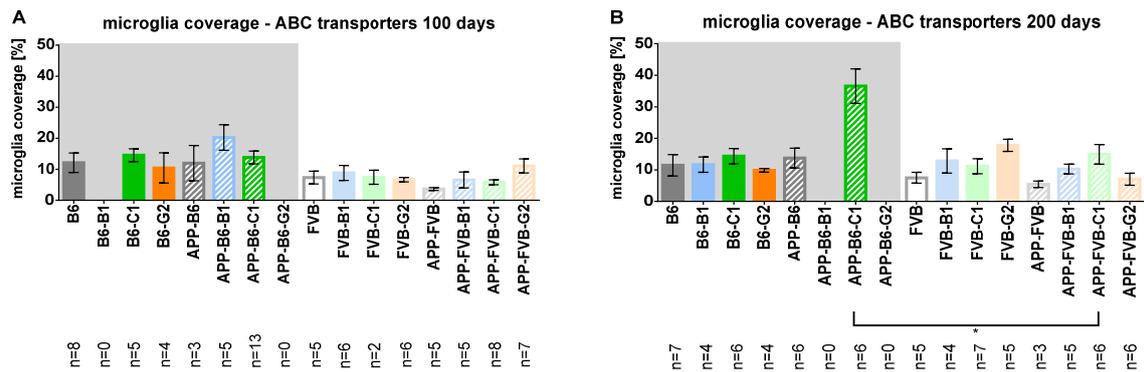
Figure 16: Effects of genetic backgrounds (B6 and FVB) on microglia coverage of mice with ABC transporter knock outs at the age of 100 (A) and 200 days (B)
200 days old APP-transgenic mice with a knockout of ABCC1 in B6 background (left, grey) show a significantly increased microglia coverage compared to FVB background (right, white). Cortical areas at Bregma +1.8mm were stained with microglia-specific antibody IBA1. 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *p≤0.05

To determine the effect of a nuclear background on microglia coverage, ABC transporter knockout strains with B6 background were compared to their equivalent non-transgenic or APP-transgenic FVB strain (Figure 16). All strains show no significant differences at the age of 100 days (A). Additionally, 200 days old non-transgenic mice show similar levels of microglia coverage of less than 25%. In contrast, 36.6% of the cortical areas of 200 days old APP-B6-C1 mice are covered with microglia. This microglia coverage is significantly higher compared to the equivalent 200 days old APP-FVB-C1 mice which show a microglia coverage of 14.9%. Contrary, 200 days old non-transgenic and APP-transgenic B6 mice with ABCB1 or ABCG2 knockout have similar levels of microglia coverage like their equivalent FVB strains (B). To sum up, regarding the background strain exclusively APP-transgenic mice with ABCC1 knockout have different levels of microglia coverage. In this case, the microglia coverage in B6 background mice is increased compared to the FVB background. Due to limitation of mouse brains no IBA1 stainings of groups 100 days B6-B1, 100 days APP-B6-G2, 200 days APP-B6-B1 and 200 days APP-B6-G2 exist.

Astrocyte coverage
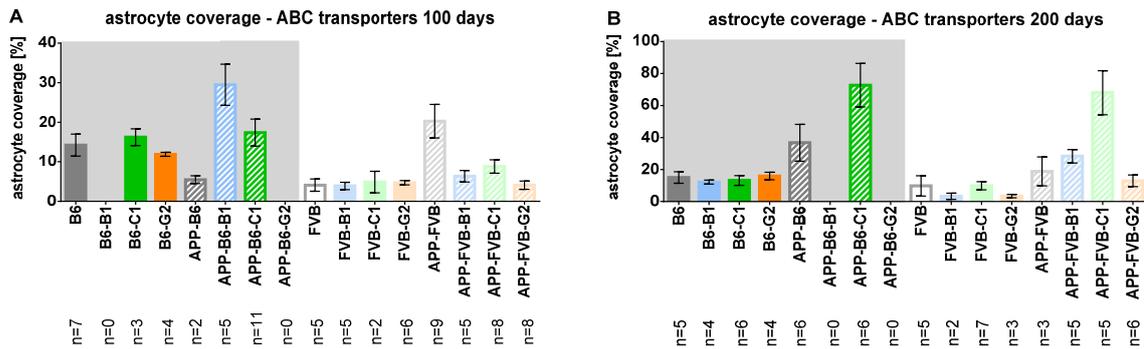
Figure 17: Effects of genetic backgrounds on astrocyte coverage of mice with ABC transporter knockouts
Genetic backgrounds, B6 (left, grey) and FVB (right, white), do not affect astrocyte coverage in cortical area of mice with ABC transporter knock outs neither at the age of 100 (A) nor at 200 days (B). Cortical areas at Bregma +1.8mm were stained with astrocyte-specific antibody GFAP. 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *p≤0.05

Non-transgenic and APP-transgenic ABC transporter knockout (ABCB1, ABCC1, ABCG2) mice with either FVB or B6 background were stained with an antibody against GFAP and examined to investigate differences in astrocyte coverage. Due to limited sample material, no GFAP stainings of 100 days B6-B1, 100 days APP-B6-G2, 200 days APP-B6-B1 and 200 days APP-B6-G2 exist.

In Figure 17, astrocyte coverages of these strains are compared regarding their different backgrounds. Significant effects of nuclear background on any ABC transporter knockout strain or control strain were revealed neither at the age of 100 nor 200 days.
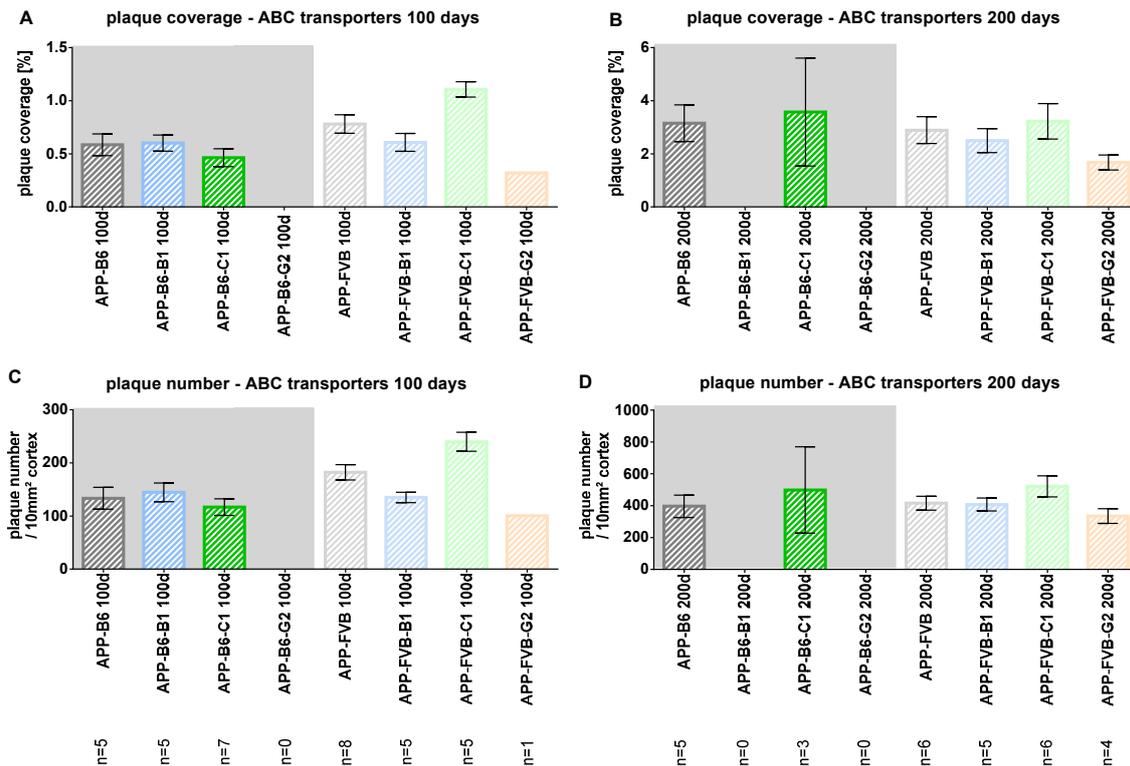
Figure 18: Effects of genetic backgrounds on plaque number and plaque coverage of mice with ABC transporter knockouts

Plaque coverage (A, B) and plaque number (C, D) in cortical area of mice with ABC transporter knockouts (ABCB1, ABCC1, ABCG2) at the age of 100 days and 200 days are independent of nuclear background B6 (left, grey) and FVB (right, white). 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *p≤0.05

To determine differences in plaque histopathology of ABC transporter knockouts with and without APP-transgene, 6F3D stainings cerebral cortices of these mouse strains were analysed. Unfortunately, stainings of the following groups exist due to sample limitations: 100 and 200 days APP-B6-G2 and 200 days APP-B6-B1.

In Figure 18, plaque coverage and plaque number of these strains are compared regarding their different backgrounds. Significant effects of genetic nuclear background on any ABC transporter knockout strain or control strain were revealed neither at the age of 100 nor 200 days.
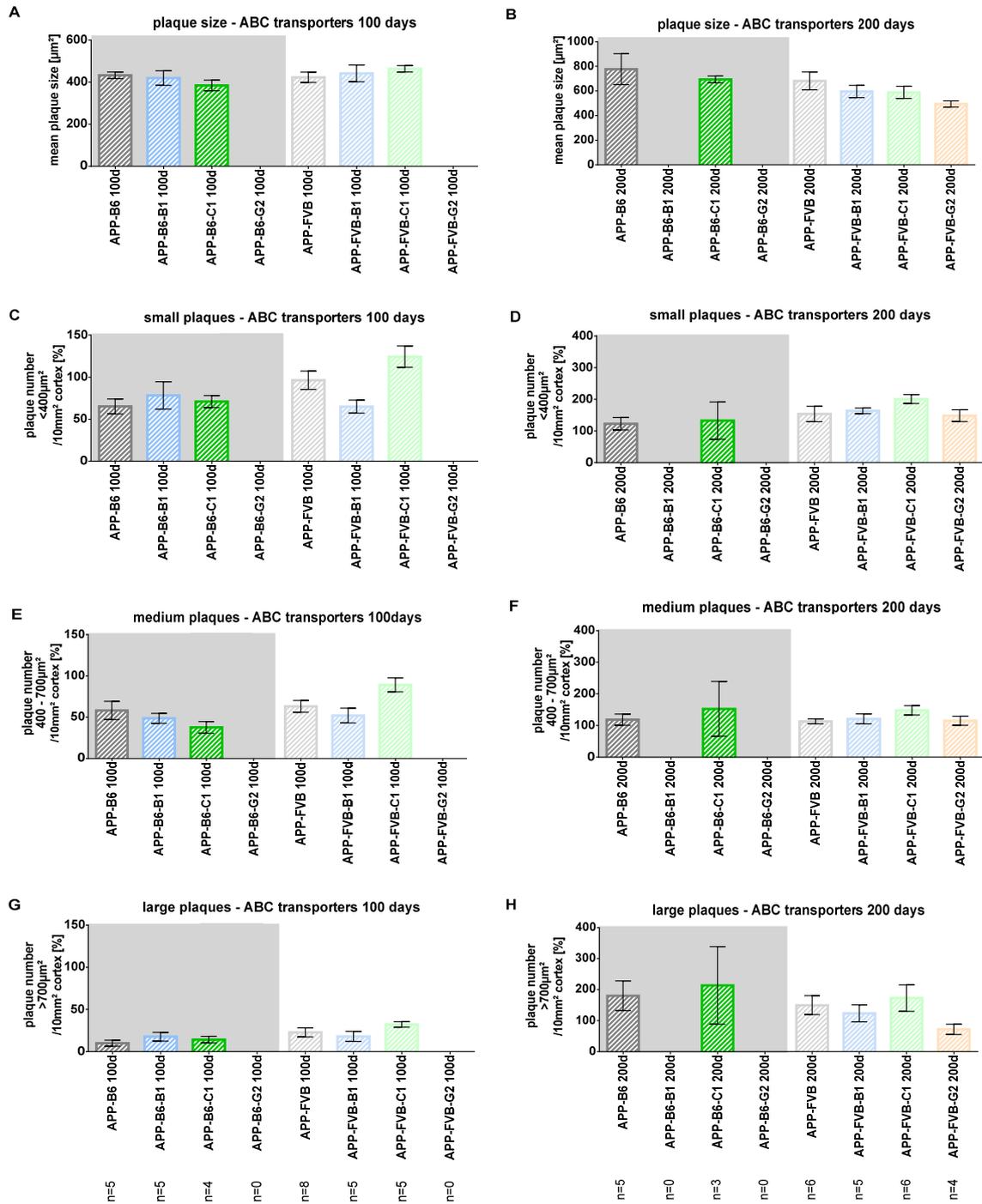
Figure 19: Effects of genetic backgrounds on plaque size (A, B) and the distribution of small (C, D), medium (E, F), and large (G, H) plaques of mice with ABC transporter knockouts
Genetic backgrounds, B6 (left, grey) and FVB (right, white), do not affect plaque size or plaque numbers of any size. Cortical areas at Bregma +1.8mm were stained with antibody 6F3D. 2way ANOVA followed by the Holm-Sidak post hoc test, *p≤0.05

Cortical areas of 100 and 200 days old APP-transgenic mice with ABCB1, ABCC1 and ABCG2 knockouts in either FVB or B6 background were stained with 6F3D. These stainings were analysed and compared to determine how nuclear background affects plaque size and the distribution of

small, medium, and large plaques (Figure 19). Due to sample limitations, no stainings of groups 200 days APP-B6-B1, 100 and 200 days APP-B6-G2 exist.

None of the nuclear backgrounds, neither FVB nor B6, affects plaque size, the distribution of small, medium, or large plaques in control strains (APP-FVB, APP-B6) or ABC transporter knockout strains (APP-B6-B1, APP-B6-C1, APP-FVB-B1, APP-FVB-C1, APP-FVB-G2).

4.2. Quantification of Aβ40 level in cerebral tissue of non-transgenic mice

The quantification of Aβ40 levels in non-transgenic mice was performed with Aβ40 Peptide (4G8) kit to check whether it is affected by genetic backgrounds, altered mitochondrial functions and ABC transporter functions. Moreover, the measurements of Aβ40 levels were performed in 100 and 200 days old mice to determine whether they have an influence on aging.

4.2.1. Influence of nuclear and mitochondrial DNA on Aβ40 level in non-transgenic mice
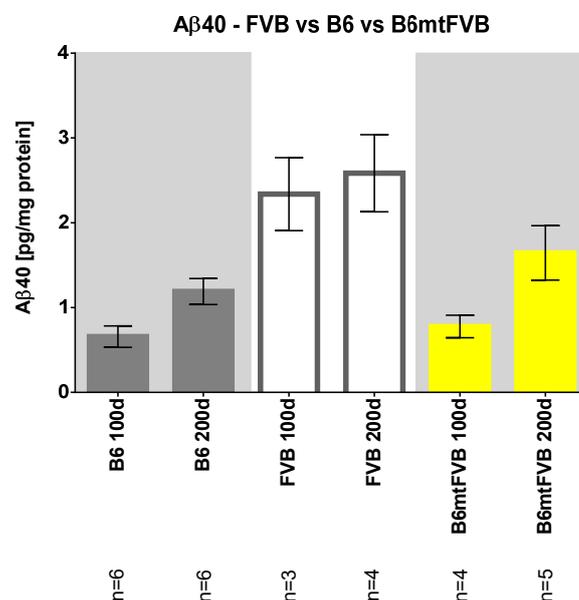
Figure 20: Effects of nuclear and mitochondrial DNA on Aβ40 levels
Neither altered nuclear background nor mutations in mtDNA affect Aβ40 levels (4G8 antibody) in brains of non-transgenic mice. Column background illustrates the nuclear background of mouse strains. (grey – B6 background; white – FVB background). 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *p≤0.05

Aβ 40 levels of 100 and 200 days old non-transgenic B6, FVB and B6mtFVB mice were measured to determine how nuclear and mitochondrial DNA affect the levels of in-mouse Aβ. Measurements revealed no significant effects of nuclear background or the origin of mitochondrial DNA on Aβ40 levels (Figure 20).

4.2.2. Influence of mutations in mtDNA on Aβ40 level in non-transgenic mice
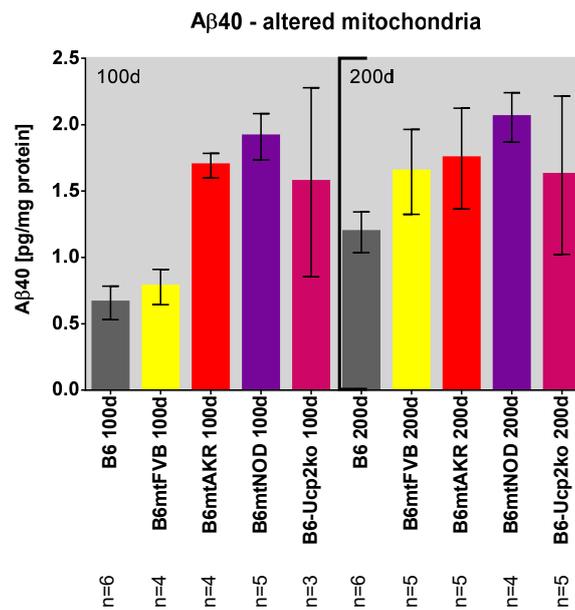
Figure 21: Effects of altered mitochondrial functions on Aβ40 levels of B6 mice
Alterations of mitochondrial function caused by variations in mitochondrial (mtFVB, mtAKR, mtNOD) or nuclear (Ucp2ko) DNA do not affect Aβ40 levels (4G8 antibody) in brains of non-transgenic B6 mice. 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *p≤0.05

The quantifications of Aβ40 levels in mouse strains B6, B6mtFVB, B6mtAKR, B6mtNOD and B6Ucp2ko were performed at 100 and 200 days to investigate whether and how modified mitochondrial functions caused by variations in mitochondrial DNA (B6mtFVB, B6mtAKR, B6mtNOD, B6Ucp2ko) affect aging. Aβ40 levels in mice with alterations of mitochondria function were measured with VPlex Aβ40 Peptide (4G8) kit. The results of these measurements showed that altered mitochondrial functions do not have an influence on Aβ40 levels neither at the age of 100 nor 200 days (Figure 21). Furthermore, the Aβ40 levels do not change significantly with increasing age, although it rises slightly in B6 and B6mtFVB mice.

4.2.3. Influence of ABC transporter knockouts on Aβ40 level in non-transgenic mice

In this section, the effects of ABC transporter knockouts ABCB1, ABCC1 and ABCG2 on Aβ40 level were quantified in genetic backgrounds B6 and FVB. In this section, the results of these quantifications are shown separately in each genetic background at 100 and 200 days.
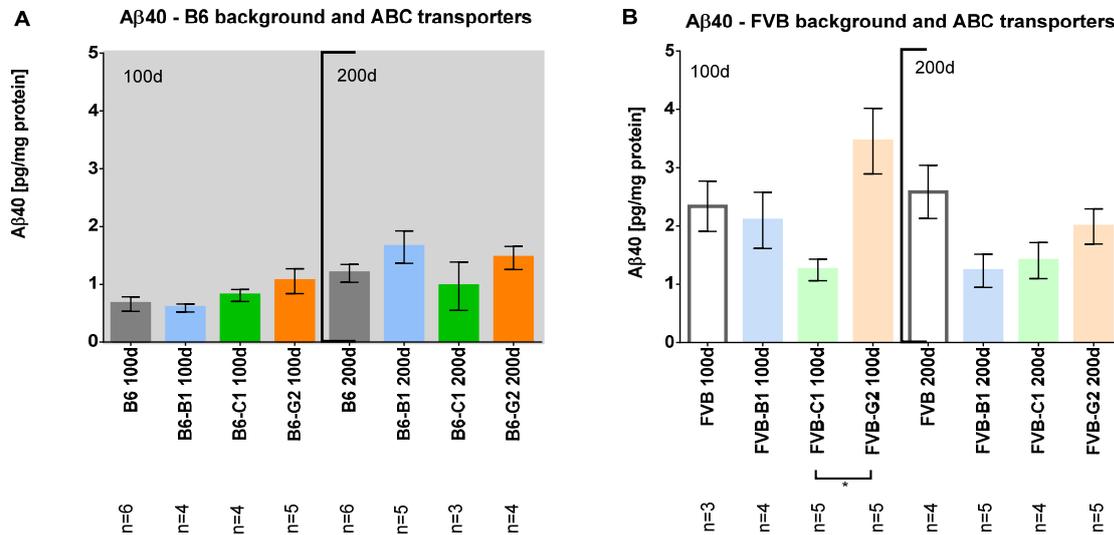
Figure 22: Effects of ABC transporter knockouts on Aβ40 levels of B6 (A) or FVB (B) background with increasing age

Aβ40 levels (4G8 antibody) in brains of 100 days old FVB-C1 mice are significantly lower than age-matched FVB-C1 mice. With this exception, Aβ40 levels of non-transgenic mice with either B6 or FVB background do not depend on ABC transporter (ABCB1, ABCC1, ABCG2) knockouts neither at 100 or 200 days nor with rising age. 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *p≤0.05

Furthermore, the influence of ABC transporter knockouts on endogenous Aβ levels was investigated by measuring Aβ40 levels in 100 and 200 days old B6 and FVB mice with ABCB1, ABCC1 or ABCG2 knockout. Analysis of these measurements showed no significant effects caused by ABC transporter knockouts compared to their control strains FVB or B6 (Figure 22). Furthermore, increasing age does not change Aβ40 levels in these strains. Nevertheless, 100 days old FVB-G2 mice have a significant higher Aβ40 level than FVB-C1 mice. This fact is not true for 200 days old mice of these strains.

4.2.4. Influence of genetic background on ABC transporter knockouts on Aβ40 level in non-transgenic mice

In this section, the effects of genetic backgrounds, B6 and FVB, on the Aβ40 levels of ABC transporter knockouts are analysed by a comparison of ABC transporter knockout mouse strains in B6 background versus equivalents in FVB background separately at 100 and 200 days.
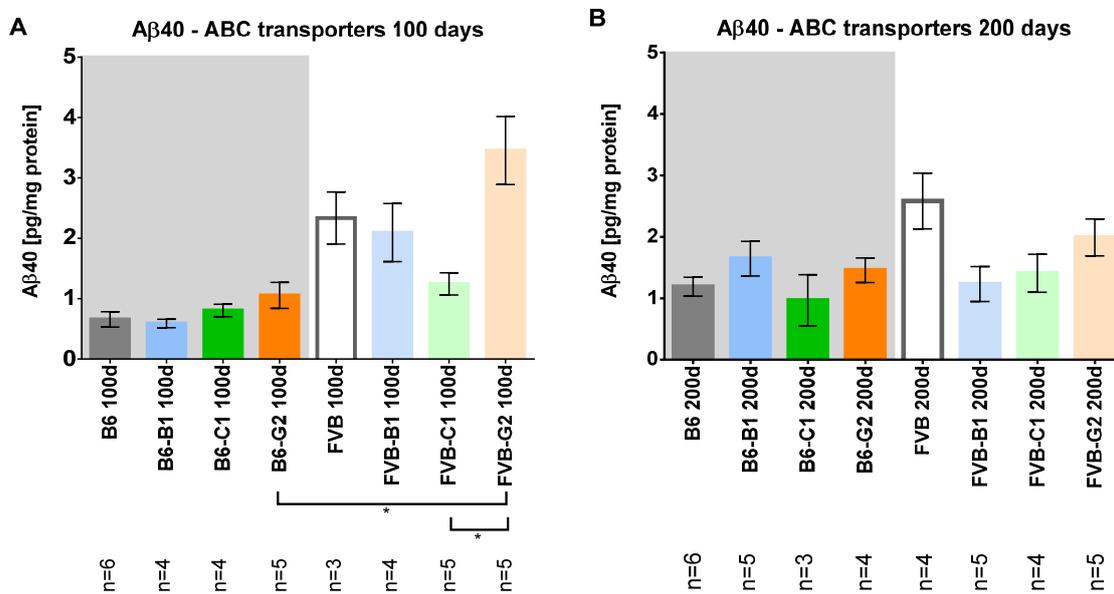
Figure 23: Effects of genetic backgrounds on Aβ40 levels in mice with ABC transporter knockouts
The knockout of ABCG2 induces a higher Aβ40 level (4G8 antibody) in 100 days old FVB mice than in age-matched B6 mice. Column background illustrates the nuclear background of mouse strains. (grey – B6 background; white – FVB background). 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *p≤0.05

Comparing the same ABC transporter knockout mouse models in B6 and FVB background revealed a significant difference between 100 days old mice with a ABCG2 knockout in B6 and FVB background (Figure 23). The Aβ40 level in FVB-G2 is approximately three times higher than in B6-G2. Furthermore, it is significantly higher than in FVB-C1 as already mentioned in the above chapter. At 200 days, the Aβ40 level of none of the ABC transporter knockout strains is affected by their nuclear background.

4.3. Quantification of buffer- and guanidine-soluble Aβ42 level in cerebral tissue of APP-transgenic mice

The quantification of buffer and guanidine soluble Aβ42 levels in APP-transgenic mice was performed with Aβ42 Peptide (4G8) kit to check whether it is affected by genetic backgrounds, altered mitochondrial functions and ABC transporter functions. Moreover, the measurements of Aβ42 levels were performed in 100 and 200 days old mice to determine whether they are influenced by aging.

4.3.1. Influence of antibody binding site on buffer and guanidine soluble Aβ42 levels in APP-transgenic mice

A comparative analysis of buffer and guanidine soluble Aβ42 levels was performed with antibodies 6E10 and 4G8 that have different binding sites. This was done to check whether these different antibodies cause diverging results.
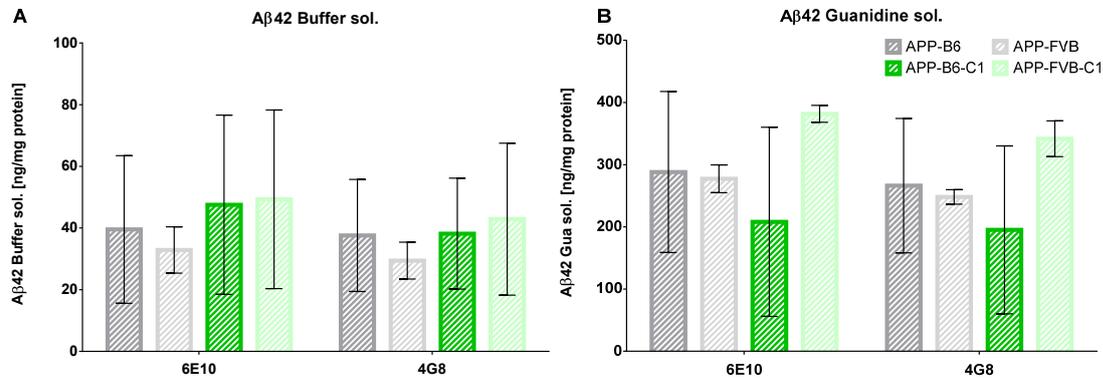
Figure 24: Binding sites do not affect buffer or guanidine soluble Aβ42 levels in APP-transgenic control or ABCC1 knockout strains.

Exemplarily, the Aβ42 levels of 175 days old mice of mouse strains APP-B6 (dark grey), APP-FVB (light grey), APP-B6-C1 (dark green) and APP-FVB-C1 (light green) were measured with two different Aβ capture antibodies (6E10 – left, 4G8 – right). These measurements were performed with the same samples but on different plates. 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *p≤0.05, n=5

Aβ42 levels of APP-transgenic control and ABCC1 knockout strains were measured with 6E10 (binds AA 3-8) and 4G8 (binds AA 18-22), respectively, to ensure that results do not depend on the chosen antibody (figure 24). The measured buffer and guanidine soluble Aβ42 levels are independent from the used antibody for all mouse strains (APP-B6, APP-B6-C1, APP-FVB, APP-FVB-C1).

4.3.2. Influence of nuclear and mitochondrial DNA on buffer and guanidine soluble Aβ42 level in APP-transgenic mice
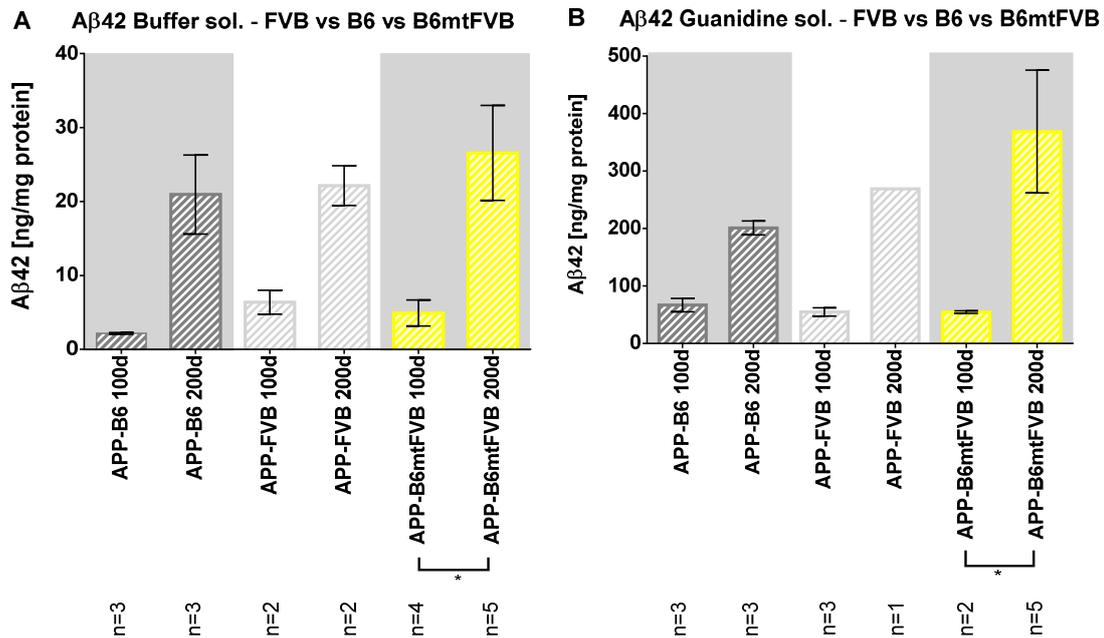
Figure 25: Effects of nuclear and mitochondrial DNA on Aβ42 levels of APP-B6, APP-FVB and APP-B6mtFVB mice

Neither different mitochondrial (mtFVB, mtB6) nor nuclear (B6, FVB) DNA affect buffer or guanidine soluble Aβ42 levels (4G8 antibody) in brains of APP-transgenic B6 mice. Column background illustrates the nuclear background of mouse strains. (grey – B6 background; white – FVB background). 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *p≤0.05

Aβ42 levels of APP-transgenic B6, FVB and B6mtFVB mice at the age of 100 and 200 days were quantified to find out whether nuclear or mitochondrial DNA affect AD pathology. Due to sample limitations, it was not possible to measure guanidine soluble Aβ42 level of 200 days old APP-FVB mice (Figure 25).

Statistical analyses of measured Aβ42 levels revealed no significant effect of either nuclear or mitochondrial DNA. This includes buffer and guanidine soluble Aβ42 levels. However, increasing age results in rising buffer and guanidine soluble Aβ42 levels in B6, FVB and B6mtFVB mice. Nevertheless, the increase is significant in APP-B6mtFVB mice.

4.3.3. Influence of alterations in mitochondrial function on Aβ42 level in APP-transgenic mice
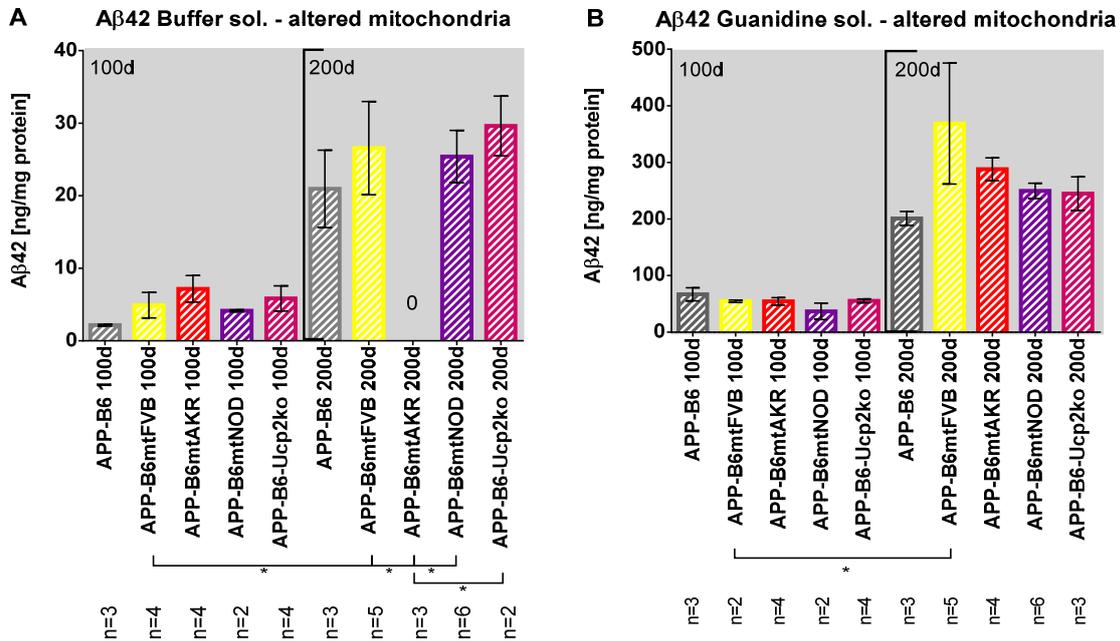
Figure 26: Altered mitochondrial function affects Aβ42 levels in APP-B6 mice
Buffer and guanidine soluble Aβ42 levels (4G8 antibody) increase with rising age in APP-B6mtFVB mice. Moreover, buffer soluble Aβ42 levels of 200 days old APP-B6mtAKR is significantly lower than in age-matched APP-B6, APP-B6mtFVB, APP-B6mtNOD and APP-B6Ucp2ko mice. 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *p≤0.05

The effects of altered mitochondria function on AD pathology were investigated by measuring buffer and guanidine soluble Aβ42 levels in APP-B6mtFVB, APP-B6mtAKR, APP-B6mtNOD and APP-B6-Ucp2ko mice.

Buffer and guanidine soluble Aβ42 levels of mouse strains with altered mitochondrial function (APP-B6mtAKR, APP-B6mtFVB, APP-B6mtNOD, APP-B6Ucp2ko) increase with rising age with one exception (Figure 26). The level of buffer soluble Aβ42 in APP-B6mtAKR decreases with rising age. The increase of Aβ42 level in APP-B6mtFVB with rising age is significant. At the age of 100 and 200 days, respectively, alterations of mitochondrial function do not affect these Aβ42 levels. This is true for comparisons between strains with changed mitochondrial function to each other and to their control strain APP-B6.

4.3.4. Influence of ABC transporters on Aβ42 level in APP-transgenic mice

In this section, the effects of ABC transporter knockouts ABCB1, ABCC1 and ABCG2 on buffer and guanidine soluble Aβ42 levels were quantified in genetic backgrounds B6 and FVB. The results of these quantifications are shown separately in each genetic background at 100 and 200 days.
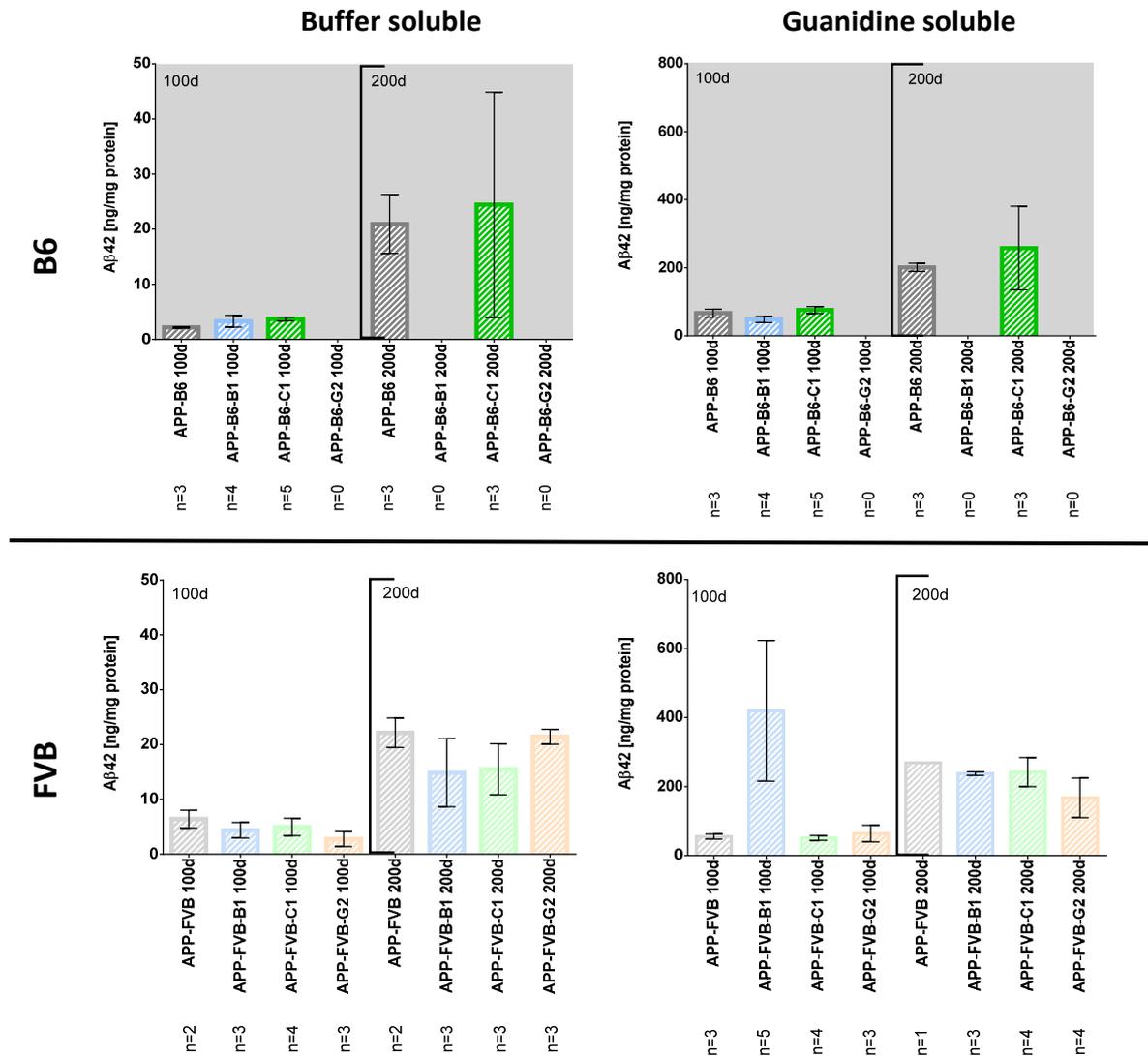
Figure 27: Effects of ABC transporter knockouts on Aβ42 levels of APP-transgenic B6 and FVB backgrounds
All ABC transporter knockouts (ABCB1, ABCC1, ABCG2) have no influence on buffer and guanidine soluble Aβ42 levels (4G8 antibody) in brains of APP-transgenic B6 and FVB mice. Column background illustrates the nuclear background of mouse strains. (grey – B6 background; white – FVB background). 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, * p≤ 0,05

Buffer and guanidine soluble Aβ42 levels are indicators visualizing the stage of AD pathology. 100 and 200 days old B6 and FVB mice with ABCB1, ABCC1 and ABCG2 knockouts were analysed to investigate the influence of ABC transporters (Figure 27). Unfortunately, buffer and guanidine soluble Aβ42 levels of 100 and 200 days old APP-B6-G2, 100 days old APP-B6-B1 mice and 200 days old APP-FVB mice have not been measured as the number of samples was limited.

At the same age, comparisons of ABC transporter knockout mice to each other as well as a comparison of them to their control strain revealed no significant differences. This fact is true independent of background and age. Furthermore, the results show an increasing Aβ42 level with rising age for all strains except APP-FVB-B1 independent of genetic background. However, these changes are not statistically significant.

4.3.5. Influence of background strains on buffer and guanidine soluble Aβ42 level in APP-transgenic mice

In this section, the effects of genetic backgrounds, B6 and FVB, on the Aβ42 levels of ABC transporter knockouts are analysed by a comparison of ABC transporter knockout mouse strains in B6 background versus equivalents in FVB background separately at 100 and 200 days.
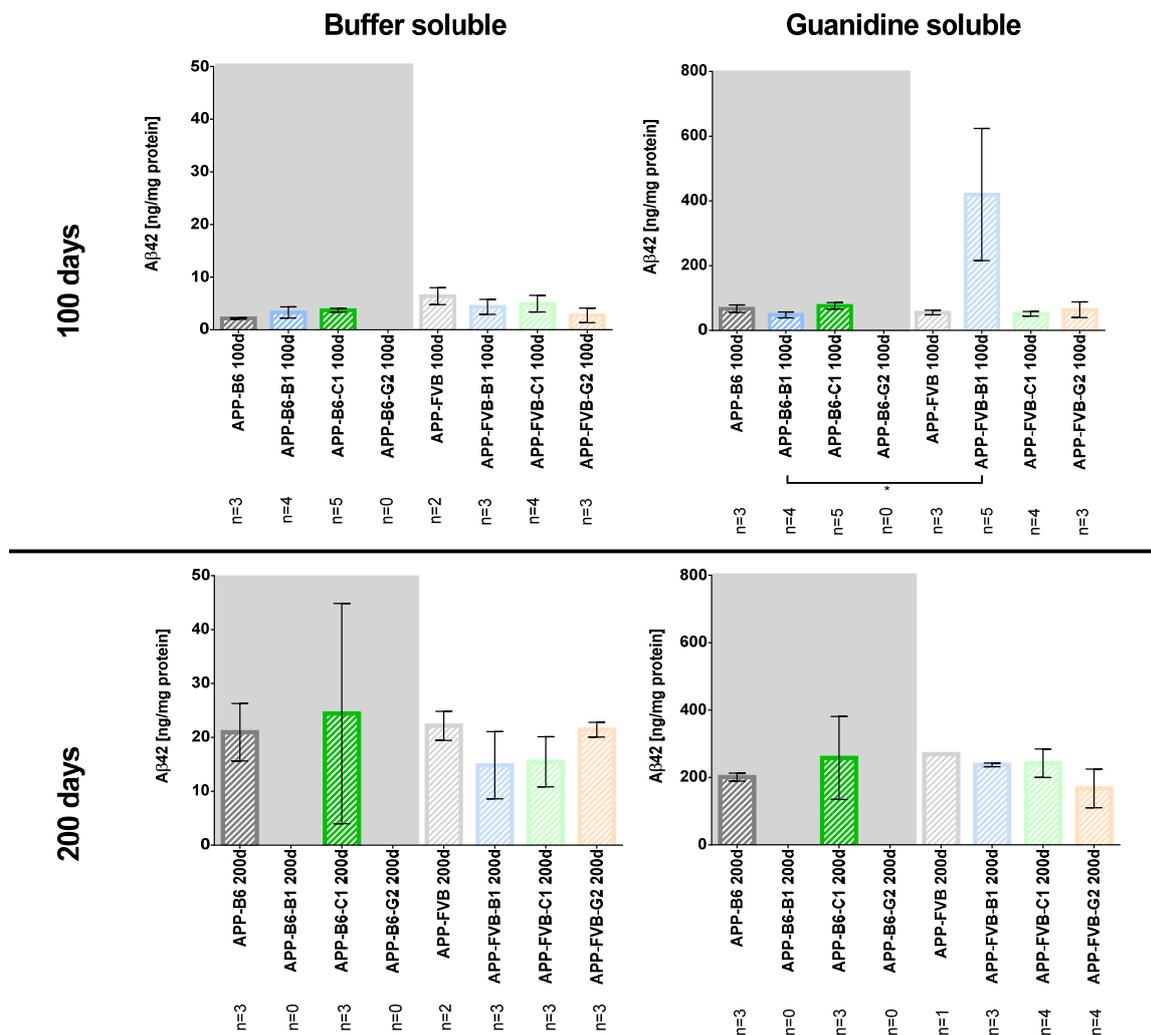
Figure 28: Influence of genetic backgrounds, B6 and FVB, on ABC transporter knockouts at 100 and 200 days
FVB background increases guanidine but not buffer soluble Aβ42 levels of in mice with ABCG2 knockout at 100 days. With this exception, neither B6 nor FVB background influence Aβ42 levels (4G8 antibody). Column background illustrates the nuclear background of mouse strains. (grey – B6 background; white – FVB background). 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *p≤0.05

Buffer and guanidine soluble Aβ42 levels of mice with ABC transporter knockouts and different backgrounds (FVB, B6) were measured. These levels were determined for 100 and 200 days old mice (Figure 28). Unfortunately, buffer and guanidine soluble Aβ42 levels of 100 and 200 days old APP-B6-G2, 100 days old APP-B6-B1 mice and 200 days old APP-FVB mice have not been measured as the number of samples was limited.

Comparing the Aβ42 levels (buffer and guanidine soluble) of 100 days old ABC transporter knockout strains with B6 background to those with FVB background revealed that the guanidine soluble Aβ42 levels of the APP-transgenic ABCB1 knockout strain is significantly higher in FVB mice than in B6 mice. Regarding the other ABC transporter knockout strains the nuclear background does not change these levels at the age of 100 days. At the age of 200 days no ABC transporter knockout strain is influenced by B6 or FVB background.

4.4. Quantification of cytokines/chemokines in cerebral tissue

4.4.1. Post-mortem stability of cytokines/chemokines
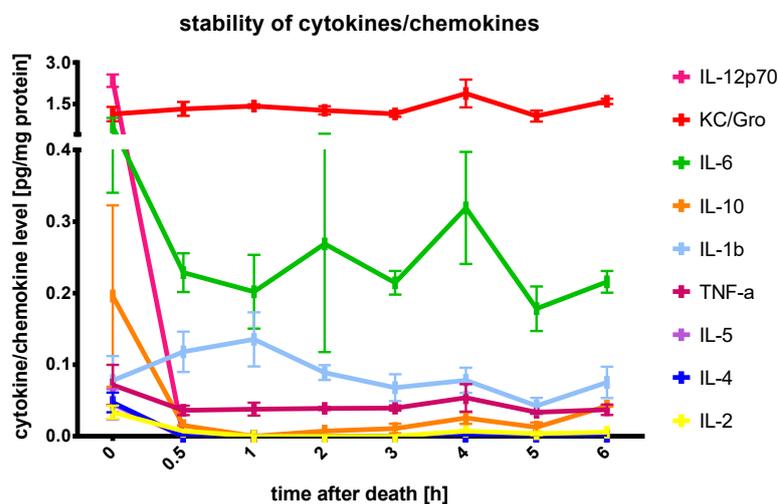
Figure 29: IL-12p70, IL-4 and IL-2 in the cortices of APP-B6 mice are degraded within the first 30 minutes after death.
100 days old APP-B6 mice were killed by cervical dislocation. Afterwards their cortices were isolated immediately (0 minutes), 30 minutes, 1, 2, 3, 4, 5 and 6 hours after death. Cortices were snap frozen in liquid nitrogen immediately after their dissection. Measurements of cytokine/chemokine levels were performed with Proinflammatory Panel 1 (mouse) kit. Multiple t-test followed by Sidak-Bonferroni test, error bars SEM, *p≤0.05, 3≤n≤6

Cytokine/chemokine levels of 100 days old APP-B6 mice were measured at different time points (0, 0.5, 1, 2, 3, 4, 5 and 6 hours) after death to determine the effect of cytokine/chemokine degradation after death (Figure 29). While the concentration of most analysed cytokines/chemokines (KC/Gro, IL-6, IL-10, IL-1b, TNF-a, IL-5) stays constant at least until 6 hours after death, the concentrations of IL-12p70, IL-4 and IL-2 decreases dramatically down to 0pg/mg protein within 30 minutes.
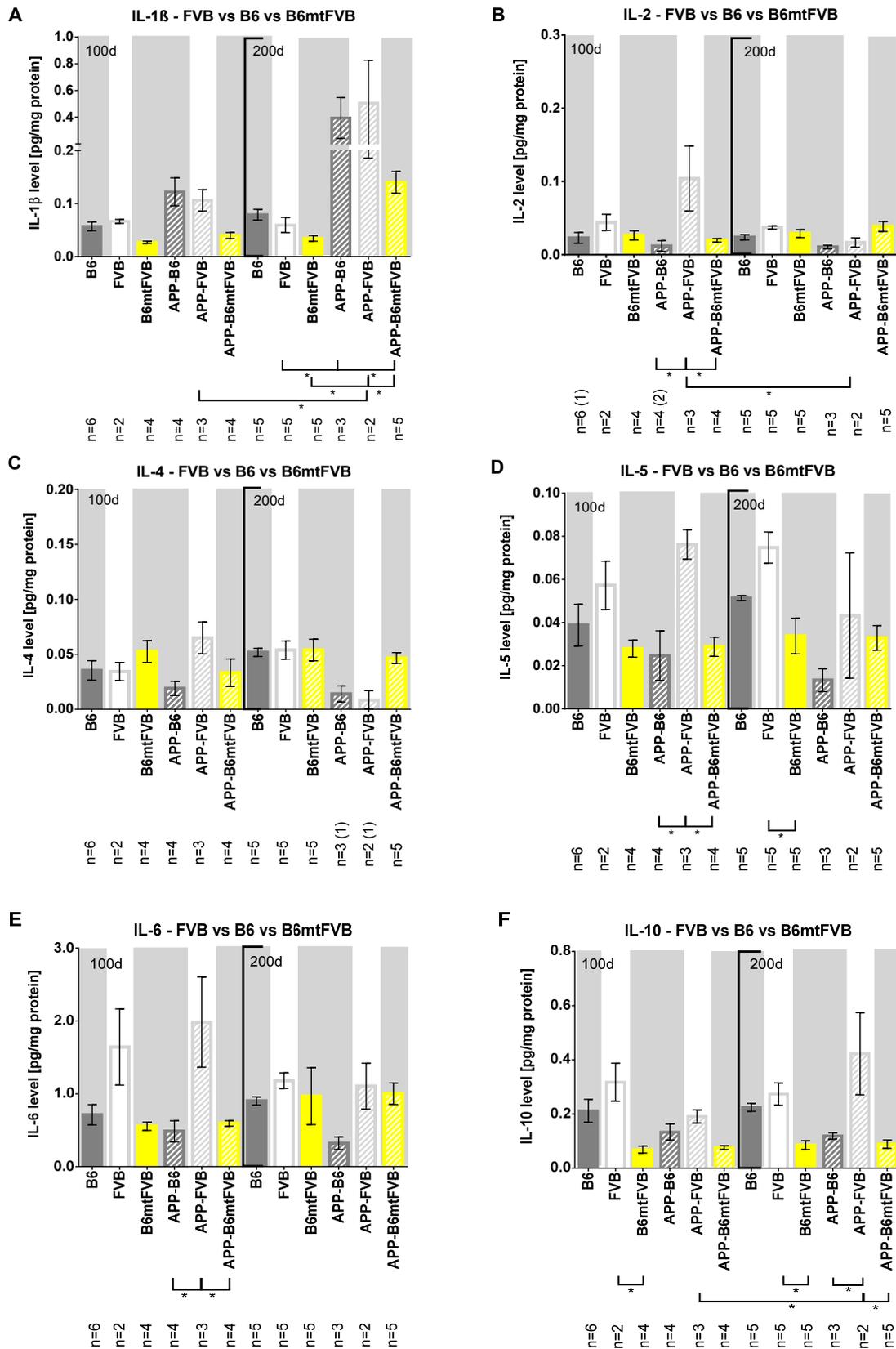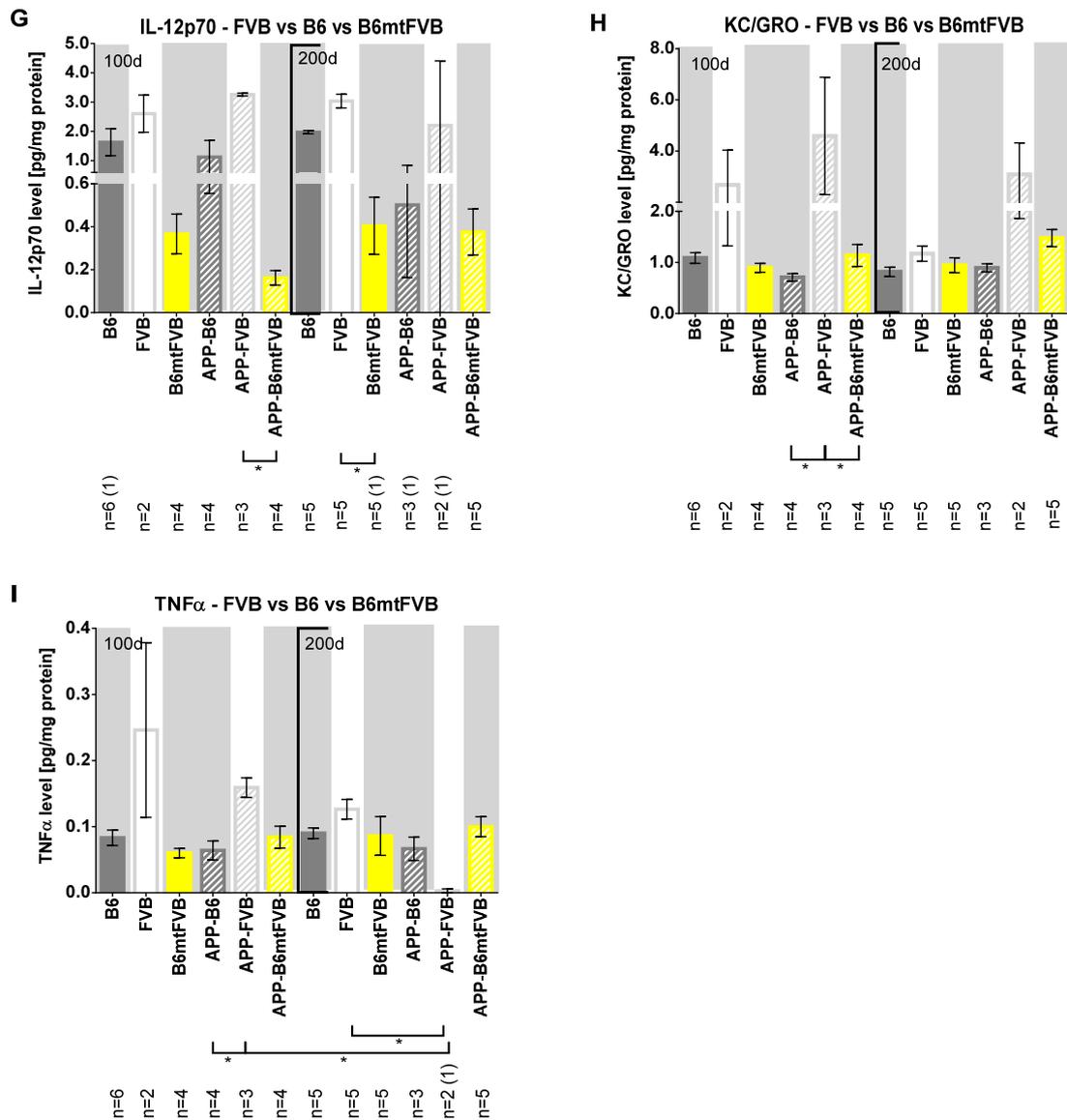
Figure 30: Influence of mitochondrial and nuclear DNA on the levels of cytokines/chemokines IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, KC/Gro and TNFα

All cytokine/chemokine levels (except IL-4) are affected by nuclear and mitochondrial DNA in 100 (left) and 200 (right) days old B6, FVB and B6mtFVB mice with and without APP-transgene. Especially, the levels of IL-2, IL-5, IL-6 and KC/GRO in 100 days old APP-FVB mice are significantly higher than in APP-B6 and APP-B6mtFVB mice. Some cytokine/chemokine levels are close to lower detection range. Therefore, some measured values are below detection range. The number of these values is written in brackets. Column background illustrates the nuclear background of mouse strains. (grey – B6 background; white – FVB background). 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *p≤0.05

A thorough comparison of cytokine/chemokine levels between all investigated strains was performed to checks the influence of nuclear and mitochondrial DNA, respectively. Therefore, background strains B6 and FVB have been compared to each other and to B6mtFVB, a strain that shares nuclear DNA with B6 mice and mitochondrial DNA with FVB mice (Figure 30). These comparisons have been performed at 100 and 200 days with and without APP-transgene.

The IL-1β levels of B6, FVB and B6mtFVB mice are similar and do not increase with rising age. Contrary, the level of this cytokine/chemokine of 200 days old APP-FVB and APP-B6 mice is

significantly higher in comparison to its 200 days old non-transgenic equivalents and 200 days old APP-B6mtFVB mice. Moreover, the IL-1β level increases with rising age in mouse strain APP-FVB.

In non-transgenic mouse strains B6, FVB and B6mtFVB IL-2, IL-6 and KC/GRO levels do not change with rising age and are not different in these strains. Nevertheless, these levels of 100 days old APP-FVB mice are significantly higher than in age-matched APP-B6 and APP-B6mtFVB mice. 100 days later, this significant difference is not apparent anymore.

The level of IL-4 is not changing significantly neither with age, APP-transgene nor between mouse strains. The IL-5 level is not altered by age in non-transgenic B6, FVB or B6mtFVB mice. While it is significantly higher in 200 days old FVB mice than in age-matched B6mtFVB mice, the IL-5 levels are not different between these strains at 100 days.

The IL-10 level of B6mtFVB mice is significantly lower than in FVB mice at 100 and 200 days. However, this level does not change with rising age in non-transgenic mice. In APP-FVB mice IL-10 level increases with rising age, while age does not affect this level in APP-B6 or APP-B6mtFVB mice. Furthermore, it is not altered in 100 days old APP-B6, APP-FVB and APP-B6mtFVB mice. In 200 days old APP-FVB mice IL-10 is significantly increased compared to 200 days old APP-B6 and APP-B6mtFVB mice.

While mouse strains B6, FVB and B6mtFVB have similar IL-12p70 levels at 100 days, they are significantly lower in mouse strain B6mtFVB at 200 days in comparison to B6 and FVB. In APP-transgenic mouse strains IL-12p70 level is not age-dependent. At 100 days APP-B6mtFVB mice IL-12p70 concentrations are significantly lower than APP-B6 and APP-FVB. This difference is not apparent at 200 days anymore.

The TNFα level is not different between B6, FVB and B6mtFVB mice neither at 100 nor 200 days. The significant differences to 200 days old APP-FVB mice have no power as the group consists of only one measurement. Therefore, the only significant difference in TNFα levels is that 100 days old APP-FVB mice display significantly higher concentrations than APP-B6 mice.

4.4.3. Comparison of cytokine/chemokine level regarding the influence of mitochondrial mutations in APP- and non-transgenic mice

Figure 31: Effects of altered mitochondrial function caused by variations in mitochondrial and nuclear DNA on the levels of cytokines/chemokines IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, KC/Gro and TNFα

Cytokine/chemokine levels (except KC/Gro) are dramatically increased in 200 days old APP-B6Ucp2ko mice. The number of measurements below detection range is written in brackets. All significant differences indicated with # are differences to mouse strain APP-B6Ucp2ko. All mouse strains have a B6 background. 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *p≤0.05

Three mouse strains with different alterations in mtDNA and one mouse strain with a knockout of Ucp2 in nuclear DNA, which causes an altered mitochondrial function, have been investigated. The cytokine/chemokine levels of mtDNA mouse strains do not change significantly with rising age (Figure 31). Furthermore, different mtDNAs do not cause differences in cytokine/chemokine levels neither in the presence or absence of APP-transgene nor at any age investigated. Contrary, all cytokine/chemokine levels except KC/GRO are extremely high in 200 days old APP-B6Ucp2ko mice. The levels of IL-1β, IL-4, IL-5, Il-6, IL-10 and IL-12p70 increase with rising age in APP-B6Ucp2ko mice. Furthermore, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10 and IL-12p70 concentrations are lower in non- than in APP-transgenic 200 days old B6Ucp2ko mice.

Compared to age matched mtDNA mice, 200 days old APP-B6Ucp2ko mice display higher cytokine/chemokine levels of IL-2, IL-4, IL-5 and IL-12p70. Additionally, in APP-B6Ucp2ko mice IL-1β, IL-10 and TNFα levels are increased in comparison to 200 days old APP-B6mtFVB. IL-6 level is higher than in 200 days old APP-B6 and APP-B6mtAKR.

4.4.4. Comparison of cytokine/chemokine level regarding the influence of ABC transporter knockouts in APP- and non-transgenic mice

The effects of ABC transporter knockouts ABCB1, ABCC1 and ABCG2 on cytokine/chemokine levels were quantified separately in genetic backgrounds B6 and FVB at 100 and 200 days.
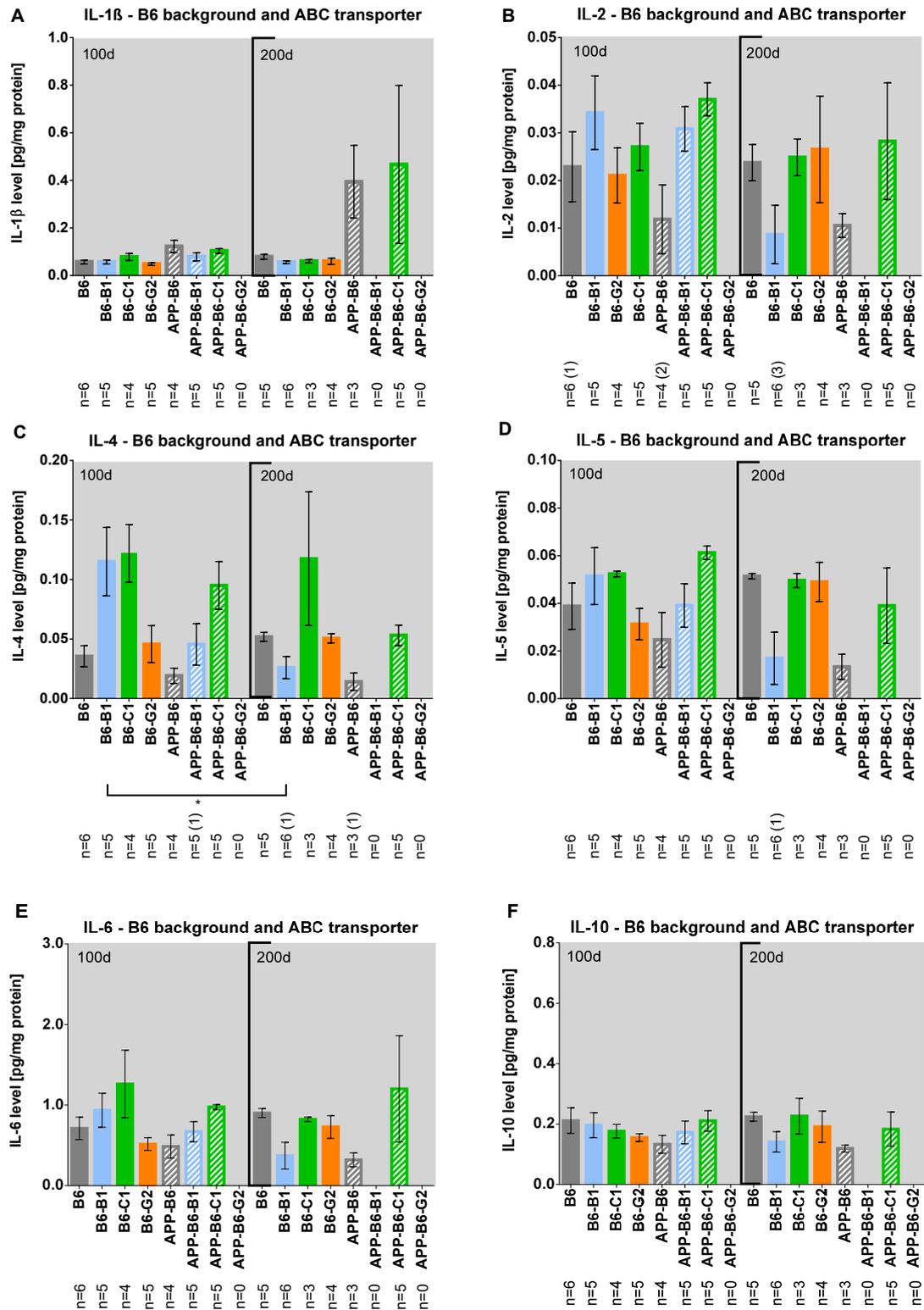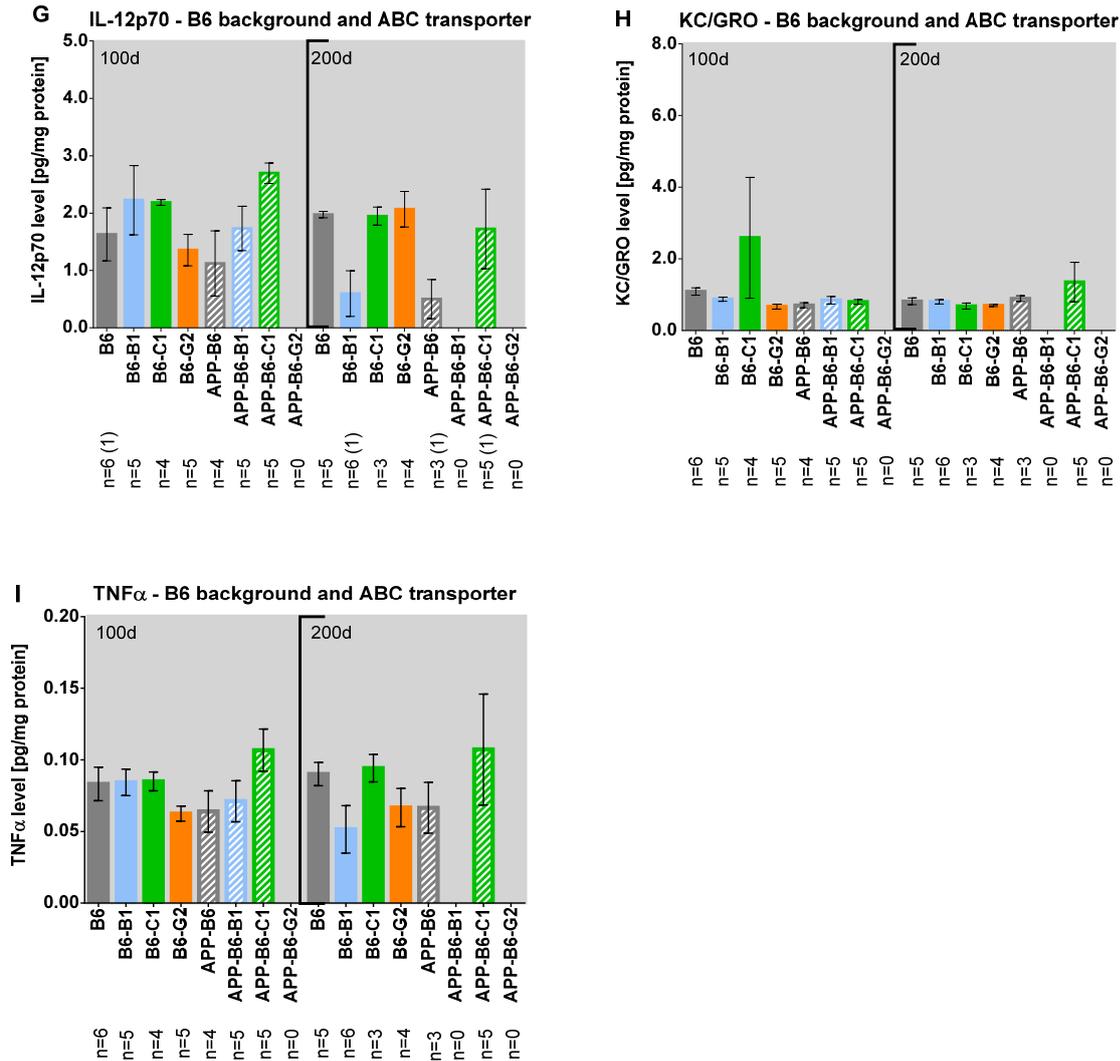
Figure 32: Influence of ABC transporter knockouts (ABCB1, ABCC1, ABCG2) on cytokine/chemokine levels of non- and APP-transgenic B6 mice
IL-4 level of ABC transporter knockout ABCB1 decreases with rising age from 100 (left) to 200 (right) days. Besides, ABC transporter knockouts do not affect any other cytokine/chemokine level at 100 or 200 days nor with rising age. The number of measurements below detection range is written in brackets. All mouse strains have a B6 background. 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *p≤0.05

One part of this investigation is to check whether cytokine/chemokine levels change in non- and APP-transgenic mice with ABC transporter knockout and whether these changes are transgene- or age-related. In B6 background, ABC transporter knockouts do not cause significant changes of cytokine/chemokine levels in comparison to their control strain or other ABC transporter knockouts (Figure 32). This fact is true for non- and APP-transgenic mice. Due to limitations of sample material no APP-transgenic B6 mice with ABCG2 knockout have been investigated. Furthermore, it has been checked whether their effects differ between non- and APP-transgenic mice. Again, no significant differences have been observed. Regarding ageing effects, statistical analyses show that the level of IL-4 decreases significantly in B6-B1 mice between 100 and 200 days. All other cytokine/chemokine levels are unaffected in B6-B1 mice.

A   IL-1ß - FVB background and ABC transporter

B   IL-2 - FVB background and ABC transporter

C   IL-4 - FVB background and ABC transporter

D   IL-5 - FVB background and ABC transporter

E   IL-6 - FVB background and ABC transporter

F   IL-10 - FVB background and ABC transporter
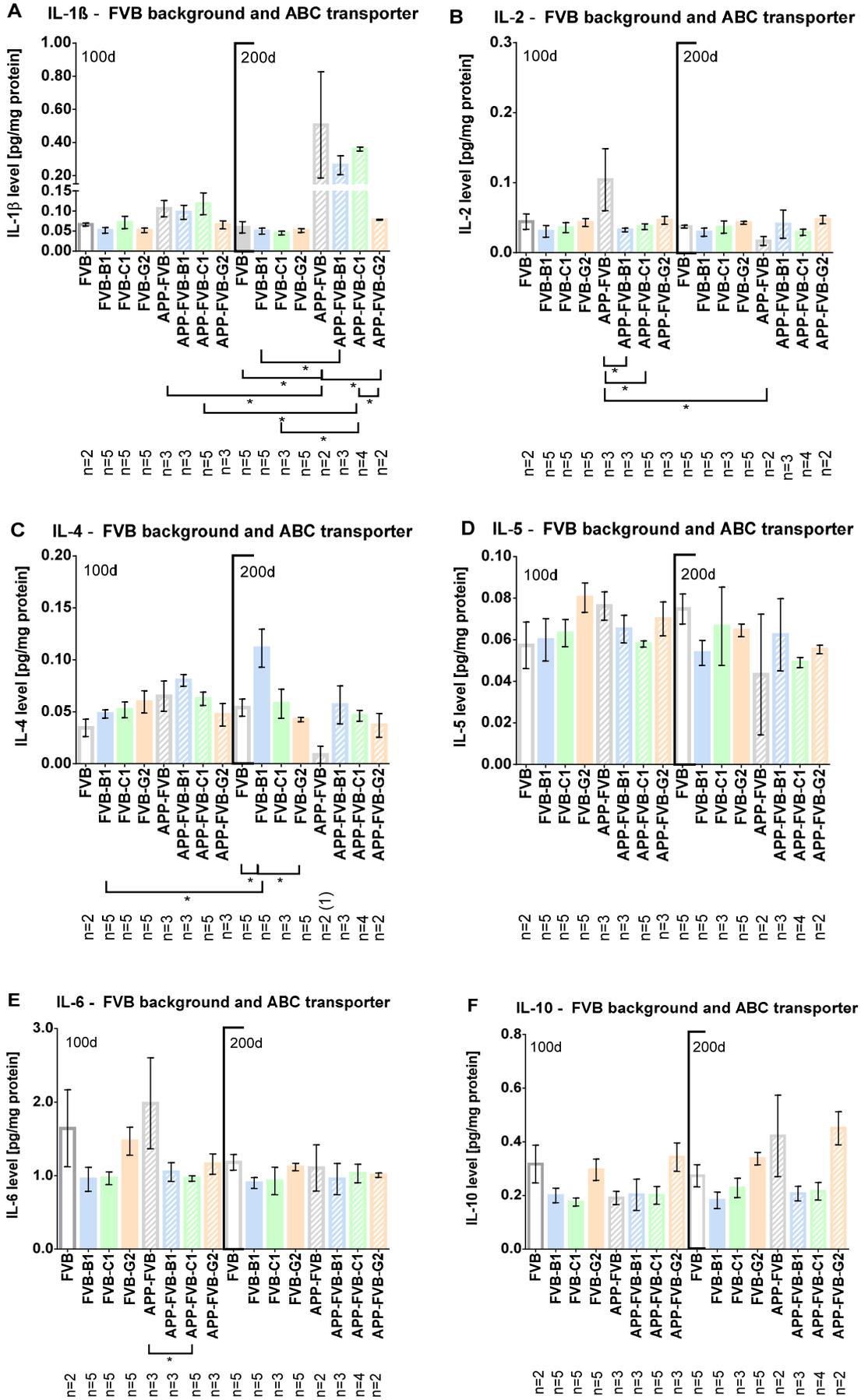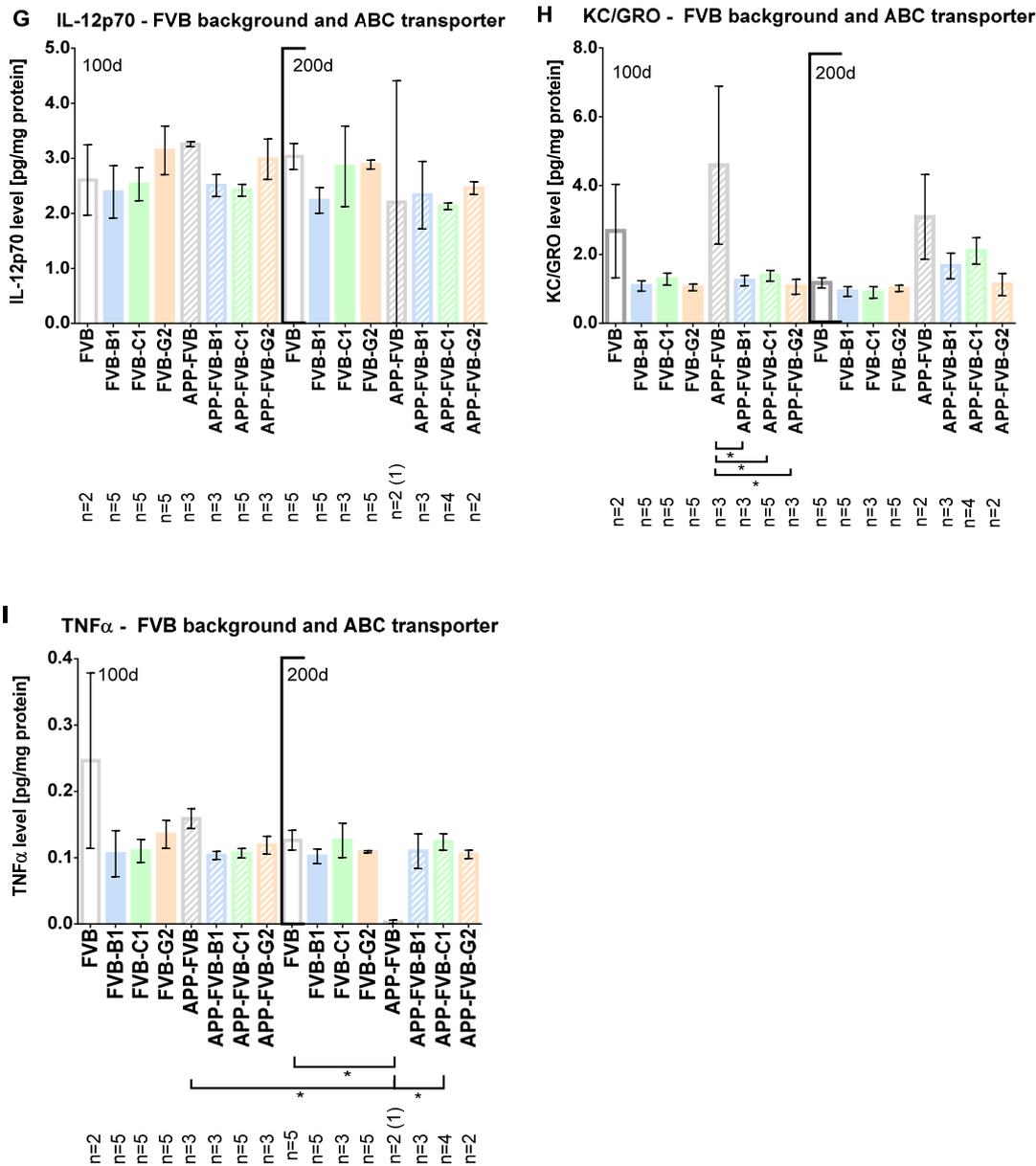
Figure 33: Influence of ABC transporter knockouts (ABCB1, ABCC1, ABCG2) on cytokine/chemokine levels of non- and APP-transgenic FVB mice

ABC transporter knockouts affect levels of cytokines/chemokines in FVB background at 100 (left) and 200 days (right). Especially, the levels of IL-2 and KC/GRO are higher in APP-FVB mice with no ABC transporter knockout than in the same strain with knockouts of ABCB1 and ABCC1. The number of measurements below detection range is written in brackets. All mouse strains have a FVB background. 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *p≤0.05

The levels of IL-5, IL-10 and IL-12p70 are not affected by any ABC transporter (ABCB1, ABCC1, ABCG2) in FVB background (Figure 33). None examined cytokine/chemokine level is affected by ABC transporter knockouts in non-transgenic FVB mice. The only exception is that IL-4 level is significantly increased in 200 days old FVB-B1 mice compared to age-matched FVB and FVB-C1 mice. This level is also higher than in 100 days old FVB-B1 mice.

The IL-1β level increases with rising age in mouse strains APP-FVB and APP-FVB-C1. Moreover, it significantly higher in APP-transgenic equivalents of FVB, FVB-B1 and FVB-C1 at the age of 200 days.

In 200 days old APP-FVB-G2 mice the concentration of IL-1β is significantly decreased in comparison to APP-FVB and APP-FVB-C1.

The IL-2 level of mouse strain APP-FVB is significantly higher than in APP-FVB-B1 and APP-FVB-C1 at 100 days. At 200 days, all APP-transgenic mice with ABC transporter knockouts have similar IL-2 levels like APP-FVB mice. In non-transgenic FVB mice neither age nor ABC transporter knockout affect the concentration of IL-2.

In contrast, the levels of IL-6, KC/GRO and TNFα are not affected by age, APP-transgene or ABC transporter knockout. The levels of IL-6 and KC/Gro each have one exception. The IL-6 concentration in 100 days old APP-FVB-C1 mice is decreased significantly compared to 100 days old APP-FVB mice. The KC/Gro concentration of 100 days old APP-FVB mice is significantly higher than in APP-transgenic mice with ABC transporter knockouts. The significant lower level of TNFα level in 200 days old APP-FVB mice is based on only one measurement in this group. Therefore, the resulting significances have no power.

4.4.5. Comparison of cytokine/chemokine level regarding the influence of genetic background in APP- and non-transgenic mice

In this section, the effects of genetic backgrounds, B6 and FVB, on the cytokine levels of ABC transporter knockouts are analysed by a comparison of ABC transporter knockout mouse strains in B6 background versus equivalents in FVB background separately at 100 and 200 days.

I IL-6 - ABC transporters 100 days

J IL-6 - ABC transporters 200 days

K IL-10 - ABC transporters 100 days

L IL-10 - ABC transporters 200 days

M IL-12p70 - ABC transporters 100 days

N IL-12p70 - ABC transporters 200 days
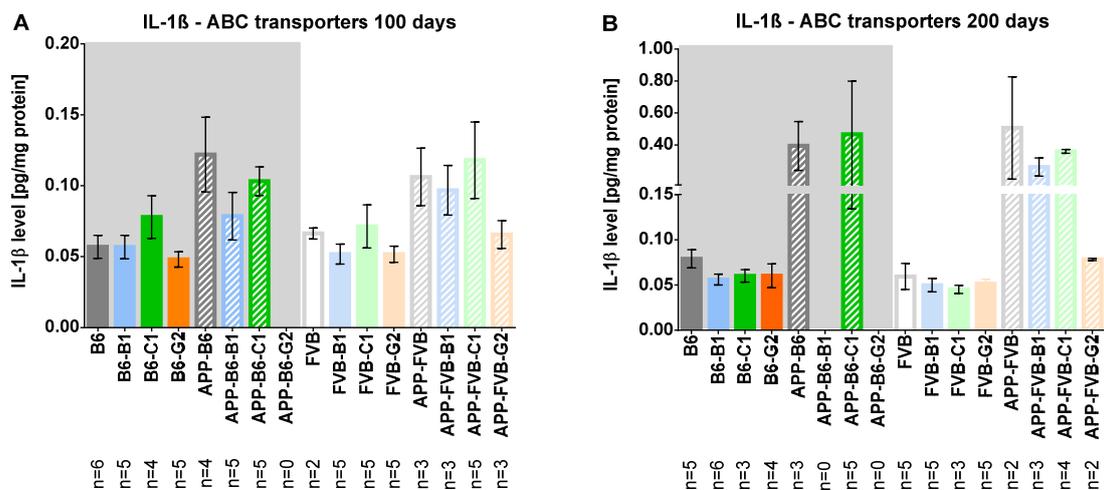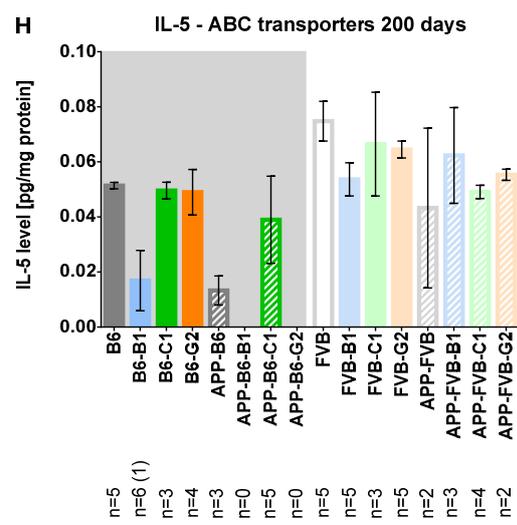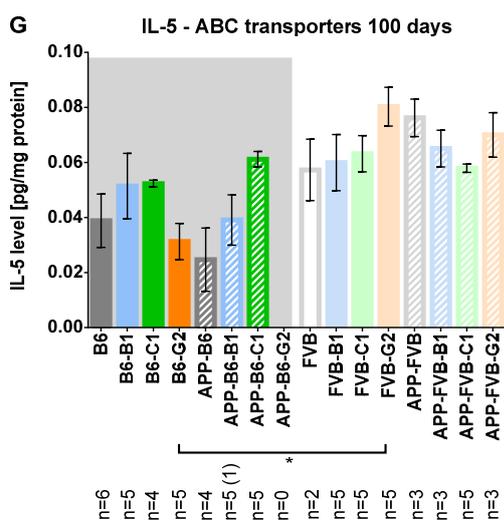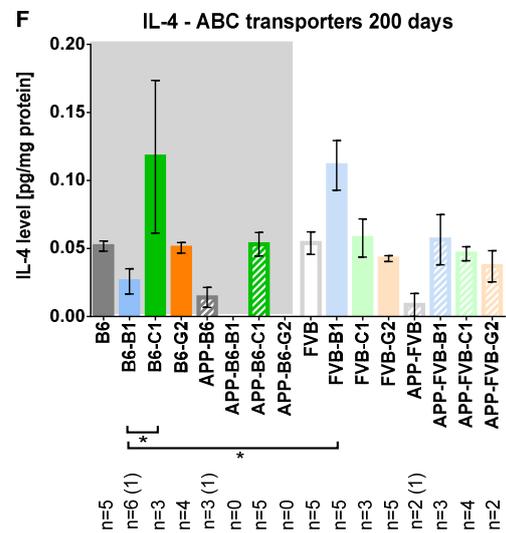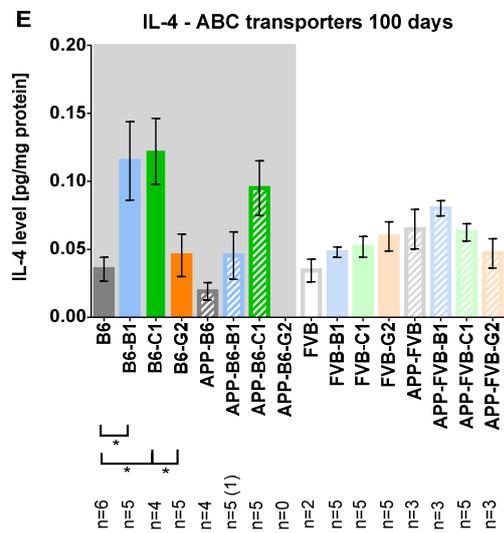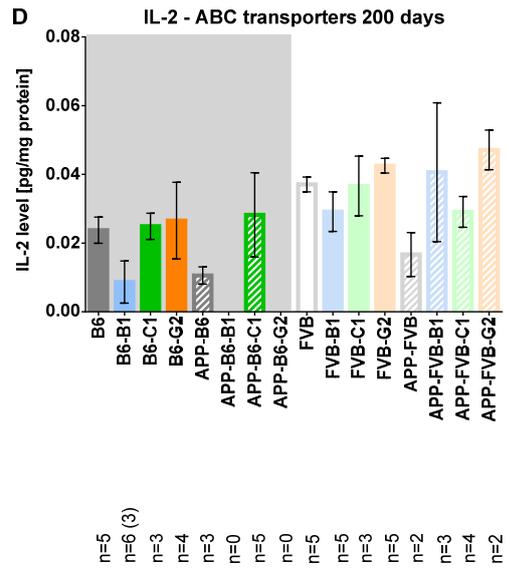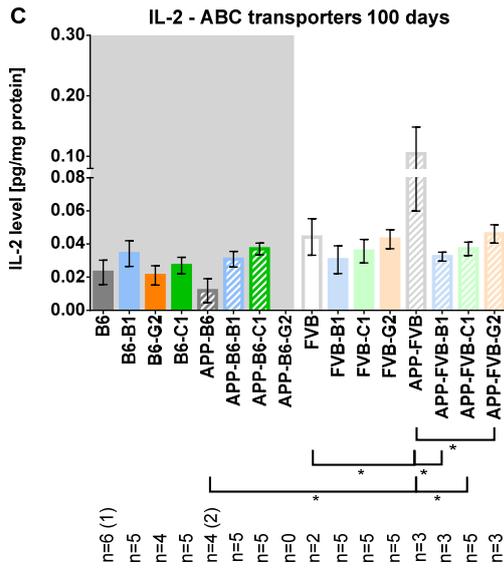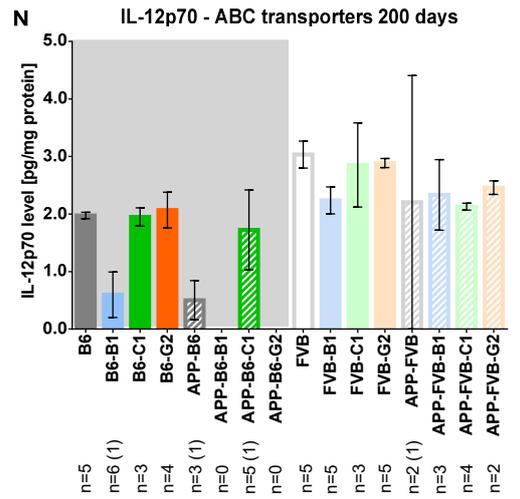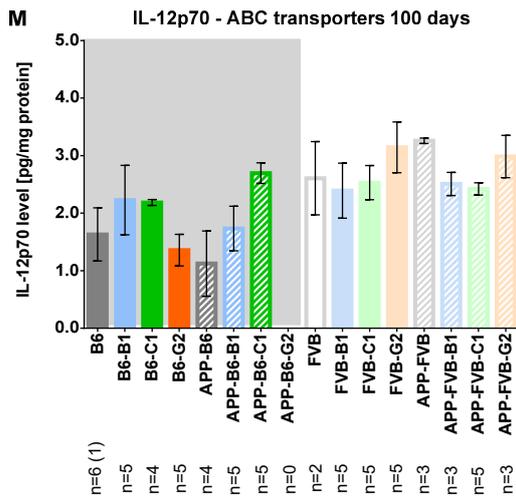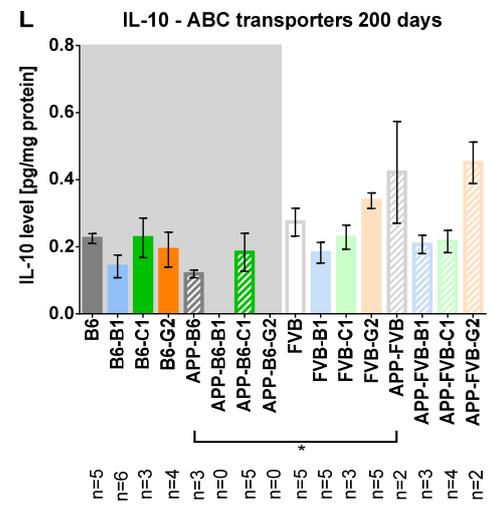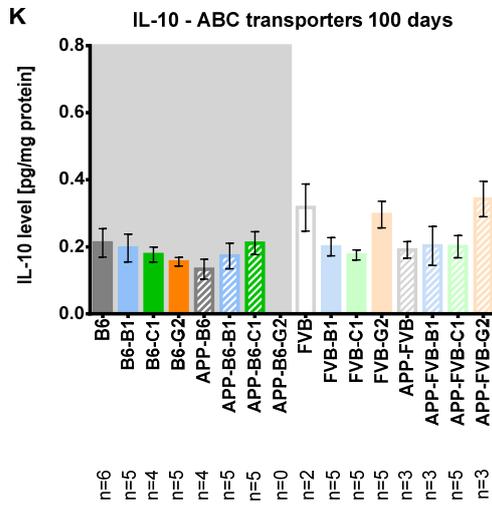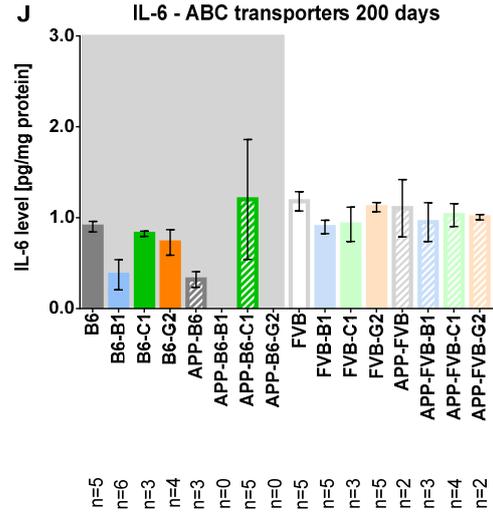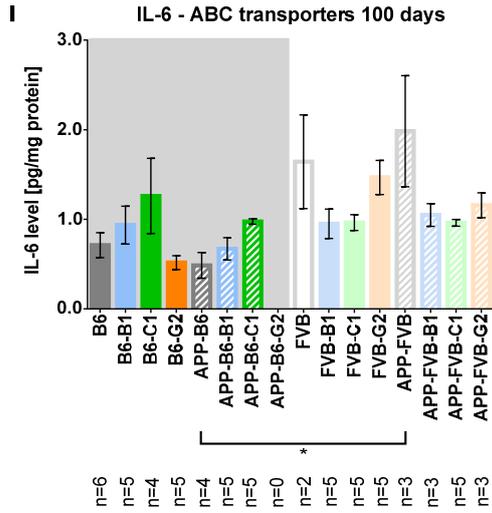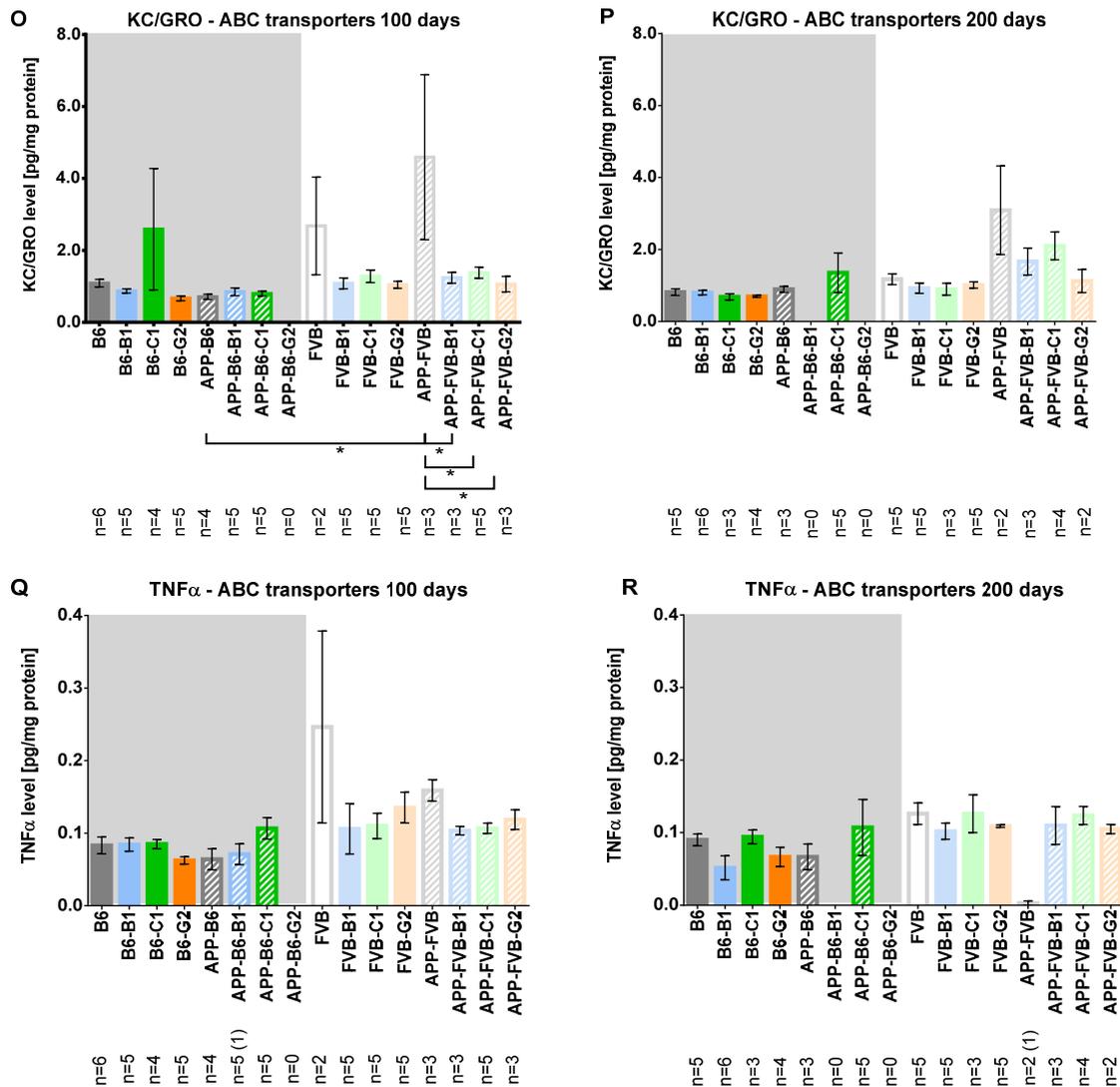
Figure 34: Influence of genetic backgrounds on cytokine/chemokine levels of mice with ABC transporter knockouts (ABCB1, ABCC1, ABCG2)
IL-4 level of ABCB1 knockout mice and IL-5 level of ABCG2 knockout mice are significantly lower in B6 than in FVB background. The number of measurements below detection range is written in brackets. Column background illustrates the nuclear background of mouse strains. (grey – B6 background; white – FVB background). 2way ANOVA followed by the Holm-Sidak post hoc test, error bars SEM, *p≤0.05

Age and APP-transgene related changes in B6 and FVB mice with ABC transporter knockouts ABCB1, ABCC1 and ABCG2 have been investigated in the previous section. In this section, the same non- and APP-transgenic strains (B6, B6-B1, B6-C1, B6-G2, FVB, FVB-B1, FVB-C1, FVB-G2) are investigated at 100 and 200 days, respectively, regarding the influence of background strains B6 and FVB (Figure 34). While in 100 days old B6-G2 mice IL-5 level is significantly lower than in 100 days old FVB-G2 mice, this difference is not apparent at 200 days anymore. However, the IL-4 level of 200 days old B6-B1 mice is significantly lower than it is in FVB-B1 mice. No other background related changes in cytokine/chemokine levels of non-transgenic ABC transporter knockout strains have been observed. In APP-transgenic mice with ABC transporter knockouts neither at 100 nor at 200 days differences in cytokine/chemokine levels have been discovered which are caused by different background strains.

5. Discussion

Alzheimer's disease has been investigated with increasing interest and effort since its discovery in 1906 by Alois Alzheimer. Therefore, symptoms, pathology, risk factors as well as different types of AD have been identified. While the causes of EOAD are well known, it is still a mystery which malfunction causes LOAD. During the last decades, investigations of AD in cell culture, mouse models and patients revealed intrinsic and extrinsic factors which contribute to the accumulation of Aβ and τ leading to malfunctions in the brain [100-103].

Researchers discuss multiple factors as cause for neurodegeneration, especially LOAD ([104]; [105]; [106]). Whereas some factors are commonly excepted, other findings like the influence of mitochondrial and ABC transporter function etc. are still controversially discussed ([55]). So far, it was not possible to identify a single reason for AD. Therefore, this investigation checks the hypothesis that AD is a multi-factorial disease triggered by a combination of factors. Within this investigation, the influence of nuclear and mitochondrial DNA, altered mitochondrial and ABC transporter function, immune status, and their relations to each other were examined as potential factors contributing to the progression of AD.

A great variety of mouse models as well as methods is needed to investigate these factors sufficiently. First, it is obligatory to choose an appropriate mouse model for AD to generate reliable results. Previous investigations revealed several differences between background strains, i.e. in behaviour, and they showed that a specific genetic background should be preferred for a certain disease model [107-111]. For example, B6 mice are a better choice to induce a chronic EAE, while SJL mice are better suited to induce a relapsing/remitting disease model [112]. So far, it has not been investigated which background strain is the better choice to model AD pathology using APP/PS1 transgenic mice. In the Pahnke lab, the mouse strain APP/PS1 is the best characterized mouse model for AD with an early formation of plaques. While previously published results of the Pahnke lab are consistent in choosing this AD mouse model, their background strain varies. This fact offers the opportunity for a comparative investigation of APP/PS1 mice in B6 and FVB background which is required urgently.

The issue of this investigation required methods characterizing plaque formation, Aβ40 and Aβ42 levels and immune states. Regarding immune status, astrocyte coverage, microglia coverage and a selection of cytokines and chemokines were analysed. The coverage of cortical area with plaques, astrocytes and microglia was investigated with immunohistochemistry. The levels of cytokines, chemokines, Aβ40 and Aβ42 were measured with electro chemiluminescent ELISAs. Unfortunately, several circumstances caused sample limitations during this work. Since most mouse models used in AD research nowadays are developed in a B6 background, the Pahnke lab decided to transfer all strains from FVB into the B6 background a few years ago. This PhD thesis has been done during this transition phase when no FVB background mice were bred anymore and the first fully backcrossed ABC transporter knockout mice became finally available. Thus, samples from ABC transporter knockout strains as well as non- and APP- transgenic mice with FVB background as well as from mouse strains with altered mitochondrial function background could not be newly produced to replace samples that had to be rejected i.e. due to incorrect genotyping. Backcrossing of mice to another background strain is very time-consuming. Thus, it would take several additional years to collect more samples of ABC transporter knock out mice with FVB background. The samples of my investigation were collected over several years. As other investigations with the same mouse strains have been performed in parallel, a limited number of samples was available especially for biochemical analyses. What will become apparent later during this discussion are the prominent differences between results from this work and data previously published by the Pahnke group. As

will be discussed now, these differences are mainly due to modifications between previous and recent methods. Therefore, questions regarding their reliability arise. For example, my analyses do not show the same significant differences between APP-B6 and APP-FVB mice as published in Fröhlich et al. [113]. Fröhlich et al. compared data which were already published in Krohn et al. and Scheffler et al. as control data [52,77]. All compared values within this publication were extracted from mentioned papers and obtained with the same methods. For example, Scheffler et al. states that plaque coverage in cortical areas of APP-B6mtFVB mice was significantly lower than in those of APP-B6 mice. However, results of the study presented here do not confirm this difference.

The failure to reproduce previous data is caused by methodological limits. This is true for immunohistochemical stainings of plaques, astrocytes, and microglia as well as quantifications of Aβ40, Aβ42, cytokines and chemokines. First, methods to stain and analyse cortical areas are the same and achieved semi-automatically in previous and recent investigations [52,77,113]. Due to semi-automatism, the results of this method do depend on only one individual decision that might lead to questionable comparability. Additionally, a repeated analysis of the exact same slides used by Krohn et al. and Scheffler et al. led to the same results as previously published (data not shown). These facts prove that the method is not the cause of differences between previous and recent data. The only step which can lead to different subjective results is the choice of cortical area that shall be analysed. Values published in Fröhlich et al. are based on data obtained by analysing cortical areas at -3.08mm and at -1.84mm from bregma. In my investigation, only one slice at -1.84mm from bregma was analysed. The results of both investigations are contrary. While Fröhlich et al. reported significant differences in the number of all, small, medium, and large plaques, these significances were not replicated in my investigation. These findings indicate that strain-specific differences appear at -3.08mm but not at -1.84mm from bregma. Consequently, it can be hypothesized that plaques spread uneven in the neocortex and that the areas of initial plaque formation are strain-specific. If further investigations will prove this fact, it would be an evidence for different plaque progression and spreading within the cortex of various mouse models. Furthermore, it would explain why results of this study are different than those in Krohn et al. and Scheffler et al.

The issues described for plaque staining also apply for analyses of microglia and astrocyte stainings. For that reason, a step-wise analysis through several brain areas or an analysis of the whole brain would be optimal in future. One promising method is CLARITY as it allows to visualize senile plaques, neurofibrillary tangles, etc. by antibody binding in the whole brain [114].

Moreover, significant differences in Aβ42 levels between APP-B6 and APP-FVB published in Fröhlich et al. could not be reproduced within my investigation. For example, in my work buffer and guanidine soluble Aβ42 levels of neither 100 nor 200 days old APP-FVB mice are significantly lower than in APP-B6 mice. In contrast, Fröhlich et al. revealed that buffer and guanidine soluble Aβ42 level of 200 days old APP-FVB mice are significantly lower than in APP-B6 mice. Furthermore, absolute values obtained with Aβ assays purchased from MSD are partly significantly lower than those published in Fröhlich et al. The quantifications of Aβ40 and Aβ42 levels are basic analyses to characterize AD mouse models. Therefore, Aβ42 levels of APP-B6 and APP-FVB mice were measured. The comparability is the most important factor to consider during establishment and standardization of Aβ42 quantification. The well-established ELISA (Enzyme Linked Immunosorbent Assay) kit manufactured by "The Genetics Company" (Switzerland) which fulfilled these requirements is not commercially available anymore. Therefore, recently these quantifications were performed with electro chemiluminescent assays purchased from Mesoscale Discovery.

However, Aβ levels measured with kits from different companies are obviously not comparable although both kits were/are approved for diagnostic purposes.

A crucial distinction between both kits were the antibodies applied for measurements. The assay from "The Genetics Company" used a WO-2 clone as capture antibody while MSD offers kits with two different clones: 6E10 and 4G8. As the quantification of Aβ40 in non-transgenic mice is part of this investigation, the inability to bind murine Aβ is a disadvantage of clone 6E10. Therefore, quantifications of Aβ40 and Aβ42 levels were performed with Aβ assays from MSD that work with clone 4G8. Clones 6E10 and 4G8 bind to different Aβ sequences. In relation to the cleavage site of α-secretase, the epitope of 4G8 is C terminal (AA18 -23) and the one of 6E10 is N terminal (AA3-8). Since this cleavage site is between these epitopes, it might cause different results. Therefore, I did a small comparison study using both kits. However, comparative quantifications of Aβ40 and Aβ42 levels within the same brain fractions of four different mouse strains (APP-B6, APP-B6-C1, APP-FVB, APP-FVB-C1) revealed that results obtained with antibodies 4G8 and 6E10 do not differ significantly (figure 24). Contrary, they even support the reliability of the method.

While sample preparation for the comparative quantification with 4G8 and 6E10 followed the instructions of Scheffler et al. and Krohn et al. [52,77], all other samples used for the quantification of Aβ40 and Aβ42 levels were prepared according to an altered protocol derived from the manufacturers protocol for cytokine/chemokine measurements. Nevertheless, the significant difference of Aβ42 levels between APP-FVB and APP-FVB-C1 mice reported by Krohn et al. was not reproduced. Therefore, these quantifications additionally prove that the altered sample preparation was not the cause of failed reproducibility in quantification of Aβ42 levels.

As mentioned, the usual sample preparation of this examination followed recommendations of Mesoscale Discovery for cytokine assays. Unfortunately, only one hemisphere was available for quantifications of Aβ40 or Aβ42 levels as well as for cytokine and chemokine levels. Therefore, the whole hemisphere planned for electro chemiluminescent ELISA was prepared as described in this protocol. This was necessary as a region-specific quantification of Aβ40, Aβ42, cytokine and chemokine levels should be avoided and thus prohibited splitting of the hemispheres before homogenization. The immunohistochemistry data already showed that results can change significantly by analysing selected areas. Unfortunately, sometimes the supernatant volume collected at the end of the protocol was too little to quantify cytokine/chemokine level and either Aβ40 level (non-transgenic mice) or buffer and guanidine soluble Aβ42 levels (APP-transgenic mice). In such cases, a decision in favour of cytokine/chemokine measurements was made. To sum up, differences between previous and recent measurements of Aβ40 levels as well as buffer and guanidine soluble Aβ42 levels seem to be caused by differences in the assays used. Therefore, another kind of assay should be used in future approaches to assess which assay reflects reality better.

In contrast to other characteristics the levels of cytokines and chemokines were not determined in previous examinations. Hence, electro chemiluminescent ELISA for quantification of cytokines and chemokines had to be established for this work. Samples for the quantification of cytokines and chemokines were prepared as recommended by the manufacturer (MSD). A big advantage of this assay is the fact that ten cytokines/chemokines can be measured at the same time with a small sample volume and it is easier to evaluate the validity of results. On the other hand, multiplex analyses can be a disadvantage as a single sample dilution easily fails to fit to all cytokine/chemokine concentrations within the sample. Within the scope of this investigation, the optimal dilutions determined in an establishing measurement did not fit to IFNγ (Figure 29). The IFNγ level of each mouse strain was below detection range. So, it is not possible to discuss the

different factors that might affect this cytokine in non- and APP-transgenic mice. IFNγ measurements might also be below detection level because it was quickly degraded after sacrifice of the mice. It is commonly assumed that cytokines and chemokines start to degrade directly after death. Therefore, the stability of cytokines and chemokines after the death of mice was examined to evaluate the reliability of obtained results. For this purpose, levels of cytokines and chemokines were quantified depending on time after death. Thus, first cytokine/chemokine quantifications were performed with brains conserved in liquid nitrogen approximately two minutes after the mouse had been sacrificed. The following timepoints of quantification were 30 minutes and 1 hour after death followed by additional measurements in one-hour steps until six hours after death. Analyses of these quantifications revealed that all analysed cytokine/chemokine levels except IL-12p70, IL-2 and IL-4 do not change significantly within the first 30 minutes. After 30 minutes, all cytokine/chemokine levels do not change anymore. Experienced researchers need about two minutes to dissect a mouse brain. Therefore, although degradation within the first 2 minutes after death could not be determined, the measurements of all cytokines/chemokines within this investigation are assumed to be reliably comparable. Nevertheless, it should be kept in mind that levels of IL-12p70, IL-2 and IL-4 are most time crucial.

In previous sections the choice of electro chemiluminescent assays to examine cytokine/chemokine levels were discussed. Additionally, the choice of analysed cytokines/chemokines and sample types should be explained. Previous investigations often revealed contrary results regarding effects of cytokines/chemokines and how they are affected in diseases etc. [115]. The conclusions of these investigations are mostly based on cell culture experiments which include only one or two cell types [116]. However, different cell types in the brain can compensate altered chemokine and cytokine production of another cell type *in vivo*. Moreover, cytokines and chemokines tend to have a great influence on each other and even more factors like disease state, brain localization, genetic factors, environmental factors etc. affect their levels [117]. Therefore, it is favourable to involve all cell types in investigations examining the correlation between a disease and cytokines/chemokines. Regarding cerebral tissue, this means that a whole hemisphere should be analysed. If analyses of whole hemispheres reveal significant differences in cytokine and chemokine levels, it is obviously not possible for any cell type to compensate alterations that might occur in one or several types. Such an extreme change will probably cause significant changes in the metabolism of the brain itself which can lead to pathophysiological changes.

Due to a very limited sample volume obtainable from each brain, the multiplex kit "Proinflammatory Panel 1 kit" from Mesoscale Discovery was the only reasonable choice for this descriptive screening study. As assay systems from other companies require more sample volume to measure a similar variety of cytokine/chemokines, they would reduce the amount of available data that help to determine issues which should be investigated in more detail. Fortunately, this kit includes the most important cytokines and chemokines that might be an issue in AD pathology. Some cytokines/chemokines do not match strictly either to pro- or anti-inflammatory functions but are rather ambivalent. It is assumed that proinflammatory and anti-inflammatory processes play a role in AD (reviewed in [118]). Especially, the concentric accumulation of microglia around plaques supports the importance of proinflammatory processes. Thus, the effects and consequences of a shifted inflammation status can be evaluated with the 'Proinflammatory Panel 1' kit to get a first overview of cytokines and chemokines and their relation to AD pathology. Later these results will be discussed in detail.

Statistical analyses of my study included at least 24 comparisons per test. Multiple t-test and ANOVA are common applicable statistical tests for analyses with more than two groups. Multiple t

tests increase the chance of false significant results. This chance rises exponentially with the performed number of t-tests. A Bonferroni correction can be used to compensate this effect. A multiple t-test with Bonferroni correction and a 2way ANOVA with Holm-Sidak correction have a similar confidence level. Due to the high power of this statistical test significant results are extremely reliable. Therefore, some differences between strains illustrated in graphs seem significant even though they are statistically not.

It appears that statistical analysis of different subjects (i.e. effect of ABC transporter knockouts on Aβ42 levels) led to different results regarding statistical significances in and between background strains. One example is that the increase of IL-10 with rising age in APP-FVB mice is significant in statistical analysis with respect to the influence of nuclear and mitochondrial DNA (Figure 30). Contrary, this change is not significant in statistical analyses regarding ABC transporter knockouts in FVB mice (discussed below, Figure 33). Differences like this are caused by comparing background strains to other strains with different scientific questions. Therefore, pure background-related changes are discussed within statistical analyses regarding the influence of nuclear and mitochondrial DNA.

I chose a 2way ANOVA with Holm-Sidak post-hoc test to compare data of the investigated mouse strains. Data of immunohistochemistry and the quantifications of Aβ40/42 levels as well as cytokines were analysed in respect of two factors, age (100 and 200 days) and mouse strain (see table 3). One might assume that the presence/absence of the APP-transgene is a third factor justifying a 3way ANOVA. However, non-transgenic strains are controls that are not relevant for the scientific question if amyloid pathology influences the measured analytes. They were strictly tested to verify that mouse models and methods worked as expected. Accordingly, 2way ANOVA is the scientifically most reasonable choice for statistical analyses.

A power test for a 2way ANOVA is not possible due to unequal sample sizes of analysed groups. Nevertheless, it can be assumed that statistically non-significant results are caused by too small sample sizes.

The previously mentioned methods and tests were executed with samples of non- and APP-transgenic mice in B6 or FVB background with and without ABC transporter knockouts or alterations in mitochondrial function. Plaque formation, the levels of Aβ40, Aβ42, cytokines and chemokines as well as astrocyte and microglia coverage were examined in several mouse strains to find out which genetic modifications affect aging and AD pathology and how they do it. The discussion of the results follows a strict outline, due to the amount of data that was generated within this investigation. In general, the discussion follows the same outline as in the results sections: influence of nuclear and mitochondrial DNA on aging and AD, effects of altered mitochondrial functions, effects of ABC transporter knockouts in B6 background, effects of ABC transporter knockouts in FVB background, and a comparison of the effects that genetic backgrounds, B6 and FVB, have on ABC transporter knockout phenotypes. Within these subtopics, non-transgenic mouse strains are compared to each other with an extra section that discusses the effects of rising age within the non-transgenic mouse strains. This is followed by a comparison of non-transgenic mouse strains with APP-transgenic equivalents. Then APP-transgenic mouse strains are compared with each other and this comparison also includes a separate discussion of age-related effects within these mouse strains.

First, control strains B6 and FVB were examined to generate data which help to interpret the data of mouse strains with a genetic modification correctly. The control strains do not differ in any examined characteristic. Therefore, it can be assumed that different genetic backgrounds do not

affect them. Additionally, they are not age-dependent within those strains. Taken together, all results indicate that nuclear backgrounds B6 and FVB do not differ in examined characteristics at any age.

The data of B6 and FVB mice are important to distinguish between effects caused by nuclear DNA and other genetic modifications. Beside the effects of nuclear DNA, it was investigated how alterations within the mitochondrial DNA affect Aβ40 levels, cytokine and chemokine levels, astrocyte, and microglia coverage. That is why B6mtFVB, a chimeric mouse strain with nuclear DNA of background strain B6 and mitochondrial DNA of background strain FVB, was characterized. The results of this characterization show that Aβ40 levels, microglia and astrocyte coverage are not affected by mitochondrial DNA. Even with rising age these characteristics do not change within B6mtFVB mice. A comparison of cytokine/chemokine levels of B6mtFVB mice and background strains shows that the IL-10 level is significantly lower in B6mtFVB mice than in 100 and 200 days old FVB mice (Figure 30). Furthermore, IL-5 and IL-12p70 levels are significantly lower in B6mtFVB mice than in FVB mice at the age of 200 days. All three cytokine levels are lower in chimeric B6mtFVB mice than in B6 and FVB mice although not reaching statistical significance at both ages. One would assume that a B6mtFVB mice either show similar values like one of the background strains or lie between those. However, the different origins of nuclear and mitochondrial DNA caused a decrease of mentioned cytokine/chemokine levels below both background strains. Therefore, it could be assumed that transcription and translation of mitochondrial genes are impaired. This impairment might be caused by a disturbed collaboration of nuclear and mitochondrial tools for the gene expression of mitochondrial DNA [119]. Nevertheless, it should be considered that the group of 200 days old FVB mice consists of only two samples which is not sufficient. So far, no other study with B6mtFVB mice exists that would explain the decreased cytokine/chemokine levels.

In the next sections, the results of a comparison between non-transgenic mouse strains B6, FVB and B6mtFVB with its matching APP-transgenic strains at both timepoints, 100 and 200 days, are discussed. An analysis of the aging effect itself within one strain is discussed in a later section. It is well known that the immune response of B6 mice is more Th1 prone, while FVB mice respond more Th2 prone to diseases [120,121]. Th1 and Th2 are two major subtypes of helper T cells which are immunity effectors. The Th1/Th2 hypothesis states that the levels of cytokines/chemokines of both subtypes are in balance in healthy subjects and that an activation of the immune system leads to an imbalance [122,123]. Therefore, significant differences in the immune response between APP-B6 and APP-FVB mice are expected.

While microglia coverage does not show any significant difference within this comparison, the astrocyte coverage of 200 days old APP-B6mtFVB mice is significantly higher than in 200 days old B6mtFVB mice (Figure 5). Although an increased coverage of cerebral area with immune cells is not sufficient to characterize an immune response, it indicates a stronger response to AD than in the other strains. This can be accompanied i.e. by an increased immune response or a tighter blood-brain barrier. The measured cytokine/chemokine levels do not support this assumption of an enhanced immune response, as these levels do not differ significantly between non- and APP-transgenic B6mtFVB mice neither at 100 nor 200 days. Nevertheless, it is possible that the level of a not examined cytokine/chemokine is significantly altered.

However, the IL-1β level is higher in APP-transgenic B6 and FVB mice than in their non-transgenic equivalents at 200 days (Figure 30). Obviously, the increased amount of Aβ in 200 days old APP-transgenic background strains causes an elevated production of IL-1β that is not apparent in APP-B6mtFVB. Wang et al. have shown that IL-1β diminishes protective effects of astrocytes and blood-

brain barrier in an *in vitro* experiment [124]. This finding does not support the results of APP-B6 and APP-FVB as it is inconsistent that mice produce IL-1β which enhances AD pathology. Even so, it might be that the immune response switches at a certain timepoint due to chronic inflammation as suggested in Scheffler et al. [99]. Furthermore, Koenigsknecht-Talboo et al. suggested that the regulative function of IL-1β on microglia is transcriptionally regulated [31]. This supports the hypothesis that the chimerism of B6mtFVB mice causes problems in transcription of IL-1β resulting in its downregulation. Indeed, their experiments also revealed that most pro-inflammatory cytokines like IL-1β suppress phagocytosis activity of microglia, which was stimulated by fibrillary Aβ. This fact indicates a detrimental function of IL-1β in AD pathology. However, the importance of further investigations is out of question, as it was reported that IL-1β levels are increased in AD patients [32,33].

Surprisingly, the TNFα level is decreased in 200 days old APP-FVB mice compared to age-matched FVB mice and does not change either in non- and APP-transgenic B6 or B6mtFVB mice. TNFα and IL-1β are known to cooperate and to induce pro-inflammatory processes i.e. phagocytosis of Aβ by microglia [125-127]. As IL-1β has similar levels in APP-FVB and APP-B6 mice, it can be assumed that the change of TNFα is independent of IL-1β. Indeed, relations between cytokines/chemokines create a complex network, which is difficult to untangle [128]. That is why several other explanations are possible. For example, one could argue that TNFα decreases exclusively in FVB background due to an inhibited production caused by a cytokine that was not quantified in this investigation [129].

So far, these findings do not indicate whether genetic backgrounds B6 and FVB respond Th1 or Th2 prone to AD. A comparable analysis of neuron numbers between non- and APP-transgenic B6, FVB and B6mtFVB mice could help to determine whether their immune responses are Th1 and Th2 prone. This is founded on the assumption that Th1 response is neurotoxic, while Th2 response is assumed to be neuroprotective. Unfortunately, such a comparable analysis was not possible with the collected samples. When sections of these samples were stained with NeuN, a neuron-specific antibody, I discovered that this staining tremendously depends on the storage duration between tissue collection and tissue embedding. Unfortunately, this time was very different between samples and therefore a comparison of neuron numbers was not possible.

As the strains analysed here do not differ significantly in most cytokine/chemokine levels, it seems as if their affinity to both types of immune response is similar. Moreover, it can be assumed that their immune response to AD is similar. Furthermore, the complex network of cytokines, chemokines and other immune regulators cannot be solved on basis of these results only and would need a separate, detailed investigation which was not within the scope of this work.

In the following sections, the characteristics of APP-B6, APP-FVB and APP-B6mtFVB mice are compared. An analysis of the aging effect itself within one strain is discussed in a later section. Significant differences between mouse strains APP-B6 and APP-FVB have been reported previously in Fröhlich et al. [113]. In addition to the analysed mouse strains in Fröhlich et al., an analysis of non-transgenic B6 and FVB mice, that were discussed before, was included in my investigation to determine whether changes in disease models are a response to AD or whether they are a characteristic of a background strain that appears no matter whether the mouse is healthy or not. Moreover, B6mtFVB and APP-B6mtFVB mice were analysed. Astrocyte coverage, microglia coverage and plaque formation do not differ between mouse strains APP-B6, APP-FVB and APP-B6mtFVB neither at 100 nor 200 days. Contrary, some cytokine/chemokine levels show differences between these mouse strains (Figure 30). For example, IL-2, IL-5, IL-6 and KC/GRO levels are significantly higher in 100 days old APP-FVB mice than in age-matched APP-B6 and APP-B6mtFVB mice. Additionally, the level of IL-12p70 is higher in 100 days old APP-FVB mice than in age-matched

APP-B6mtFVB mice. The TNFα level of 100 days old APP-FVB mice is significantly higher than 100 days old APP-B6 mice. 100 days later all these cytokine levels are not different anymore. Instead, at 200 days IL-10 level is strongly increased in APP-FVB mice in comparison to APP-B6 and APP-B6mtFVB. These results indicate that mice with a FVB background respond with more diverse changes than B6 mice and these changes caused by Aβ accumulation appear at 100 but not at 200 days. The increase of IL-10 with rising age in APP-FVB mice is consistent with other published data. It was demonstrated that increased IL-10 levels are associated with decreased Aβ uptake by microglia uptake [34,35]. Although plaque load, Aβ42 levels and microglia coverage in 200 days old APP-FVB animals are not increased in comparison to mouse strains with lower IL-10 levels, APP-B6 and APP-B6mtFVB, their phagocytosis activity should be compared. However, IL-10 levels and according phagocytosis activities of APP-B6 and APP-B6mtFVB mice measured in Scheffler et al. support this hypothesis [77].

Still, these findings do not prove that mice with genetic background B6 respond more Th1 prone and mice with genetic background FVB more Th2 prone to AD. An additional analysis of all known cytokines and a quantification of neurons should be considered to decide, whether this hypothesis is true. Nevertheless, these data help to find out, whether the immune system interacts with other contributing factors (i.e. ABC transporters) for the genesis of AD. It is obvious that further investigations are required to describe the role of IL-10 in AD pathology, especially as it was demonstrated that IL-10 level is increased in AD patients [130]. To sum up, the comparison of APP-B6, APP-FVB and APP-B6mtFVB mice reveals that APP-transgene affects cytokine/chemokine levels differently in these mouse strains and that these differences have no effect on AD pathology. Contrary to the results of Fröhlich et al., all other examined characteristics are not significantly different between these strains.

Analyses published in Fröhlich et al. are limited to comparisons of double stainings (IBA1, 6F3D) as well as quantifications of Aβ42 levels and revealed many results that were not reproduced within this investigation. Fröhlich et al. state that plaque number increases significantly with rising age in APP-B6 and APP-FVB mice. Even though one result of my investigation is a rising plaque number with increasing age in these mouse strains, these increases are not significant (Figure 6). In contrast to findings published in Fröhlich et al., 200 days old APP-FVB mice do not have significantly lower plaque numbers than APP-B6 mice. Furthermore, the distribution of plaques based on their size was not different between strains within my examination. In addition, differences in Aβ42 levels between both mouse strains were not reproduced (Figure 25). As discussed in detail before, modified methods are the most likely cause of this lack of reproducibility. Moreover, published results regarding microglia coverage are not comparable with those in this investigation. Fröhlich et al. published results based on double stainings which are slices that have been stained with two antibodies. First, antibody IBA1 binds to microglia then 6F3D binds Aβ. Afterwards this double staining was used to count plaques that are covered by microglia with more than 50%. This staining is not possible anymore, because the detection reagents used for this double staining are not commercially available anymore. No satisfying alternative could be found yet. Thus, in my investigation the ratio of a cortex area (-1.84 bregma) covered by microglia was measured and it shows that microglia coverages of cortices in non- and APP-transgenic B6 and FVB mice are not altered in different background strains or by AD. This suggests that microglia are not recruited or amplified during Aβ pathology in mice, but redistributed.

A comparison of 100 days old APP-B6, APP-FVB and APP-B6mtFVB mice with their 200 days old equivalents showed that plaque size increases significantly with age in both APP-transgenic background strains, APP-B6 and APP-FVB. Progressive AD pathology accompanied by progressive

accumulation of Aβ causes this increase. None of the other characteristics of plaque formation raises significantly with rising age in APP-B6 and APP-FVB mice. Contrary, plaque coverage and the number of all, small, medium, and large plaques increase with rising age in APP-B6mtFVB mice, while mean plaque size is not changed (Figure 9). Taken together, these results indicate that in APP-B6mtFVB mice plaque number of all sizes rises stronger than in APP-B6 and APP-FVB mice while the average plaque size does not rise in APP-B6mtFVB animals only. Additionally, buffer and guanidine soluble Aβ42 levels rise significantly between 100 and 200 days in APP-B6mtFVB mice only.

Overall, these findings reveal that plaque development is statistically different in mouse strain APP-B6mtFVB than in APP-B6 and APP-FVB although the trend is the same for all strains. While progression of plaque development in APP-B6mtFVB mice is rather characterized by the formation of new plaques, in APP-B6 and APP-FVB mice the growth of plaques is more prominent than the formation of new ones.

In strains APP-B6, APP-FVB and APP-B6mtFVB microglia coverage does not change with rising age. However, astrocyte coverage rises with increasing age in APP-B6mtFVB mice. Again, these differences seem to be caused by the chimerism of APP-B6mtFVB mice. It is not clear whether the preference of APP-B6mtFVB mice to rather form new plaques is caused by rising astrocyte coverage or vice versa, or another modification caused by chimerism. However, it is possible that the preference to form new small plaques causes increased astrocyte coverage, as plaques are numerous small inflammation foci which cause an intense inflammation response. Obviously, increased astrocyte coverage and the inhibited growth of existing plaques are associated in APP-B6mtFVB mice. It can be hypothesized that Aβ induces the secretion of cytokines/chemokines by astrocytes which leads to an inhibition of plaque growth. However, none of the examined cytokine/chemokine levels changes with increasing age in APP-B6mtFVB mice. So, it can be assumed that this lack of immune response leads to inhibited plaque growth. Alternatively, it is possible that increased astrocyte coverage and the formation of new plaques are caused or accompanied by one of the non-measured cytokines/chemokines.

No cytokine/chemokine level of APP-B6 and APP-B6mtFVB mice changes with increasing age while APP-FVB mice show age-related level changes of IL-1β, IL-2, IL-10 and TNFα. Whereas levels of TNFα and IL-2 decrease with rising age, IL-1β and IL-10 level increase. These results indicate that nuclear genetic background of FVB mice leads to a stronger immune response with rising age to AD than B6 background. Increases of IL-1β and IL-10 levels are not surprising as several previous studies have reported that IL-1β level is elevated in AD and IL-10 worsens AD pathology [33-35, 131]. Recently, it was discussed whether blocking of IL-10 is a potential therapy target (reviewed in [132]). The fact that IL-10 level does not rise in APP-B6 mice with increasing age demonstrates that genetic background has a strong influence on levels of pro- and anti-inflammatory cytokines. Therefore, it is likely that a blocking of IL-10 results in inconsistent effects. Furthermore, a manipulation of a single cytokine/chemokine out of a complex network of cytokines might be too simplistic.

Furthermore, many researchers hypothesize a change of inflammatory status with progressing AD pathology from pro-inflammatory (Th1) to anti-inflammatory (Th2) response [133]. Th1 response is effective against intracellular pathogens and Th2 against extracellular pathogens. Based on this knowledge the switch from Th1 to Th2 response would be a logical consequence in AD. In an early stage of amyloidosis, the response of immune cells in the brain is Th1 prone as Aβ is formed within neurons first and later Aβ is released by dying neurons and causes a switch to Th2 response.

Overall, age-related results do not support the hypothesis that inflammatory status changes during AD pathology from pro-inflammatory to anti-inflammatory when assessed in whole brain homogenates. Cytokine/chemokine levels are not age-related in APP-B6 or APP-B6mtFVB mice. On the other hand, even altered cytokine/chemokine levels in APP-FVB do not back the hypothesis as decreasing Th1 cytokine levels, IL-2 and TNFα, diminish proinflammatory response with rising age, while rising IL-10 level, a Th2 cytokine, balances Th1/Th2 response. Unfortunately, changes determined in cytokine/chemokine levels of APP-FVB mice might not be representative as the group of 200 days old APP-FVB consists of only two animals. Eventually, the hypothesis of a changing inflammatory status needs an analysis of all cytokines/chemokines to be proven right or wrong.

To sum up, all characteristics of non-transgenic mice are similar in B6 and FVB mice, even with rising age. Contrary, the responses of B6 and FVB mice to AD differ significantly. However, the hypothesis of a more Th1 prone response in B6 and more Th2 prone response in FVB mice cannot be supported. Furthermore, it cannot be determined whether FVB or B6 background respond stronger to amyloidosis. Nevertheless, it is obvious that the immune state changes significantly from 100 to 200 days in APP-FVB mice but not in APP-B6 mice. Extensive comparisons of mouse models that do not simulate diseases are not common in research. Nevertheless, the mentioned data regarding B6, FVB and B6mtFVB mice will help to differ between changes caused by nuclear DNA, aging, or examined disease. Many previous comparative investigations of B6 and FVB mice led to the conclusion that these strains respond different to a variety of diseases. This examination does not demonstrate distinct immune responses to AD in the brain which are expected for B6 and FVB mice. The chimerism of APP-B6mtFVB mice causes a significant different plaque development than in APP-transgenic background strains B6 and FVB. Based on results of my investigation, it is likely that an altered immune response is not the initial cause for this difference since these are minor. Overall, these results show that B6mtFVB chimerism alters progression of Aβ accumulation stronger than nuclear DNA.

The next question of this investigation was to check whether other types of chimerism in B6 and APP-B6 mice cause similar differences. To this end, non- and APP-transgenic B6mtFVB, B6mtAKR and B6mtNOD mice were examined and compared to each other. All these mice have nuclear DNA of a B6 mouse, while their mtDNA contains genetic varieties compared to mtDNA of B6 mice (table 1). Additionally, mouse models with a knockout of Ucp2, a protein encoded in nuclear DNA, were analysed. This knockout reduces mitochondrial membrane potential. Mice with B6 background and Ucp2ko were examined to check whether altered mitochondrial functions caused by nuclear genetic modifications and by chimerism have the same effects [77].

Before analysing the effects of altered mitochondrial functions on AD, their influence on non-transgenic B6 mice was examined. In comparison to B6 mice neither mitochondrial mutations nor a nuclear knockout of Ucp2 caused any significant changes in Aβ40 level, cytokine levels, astrocyte coverage or microglia coverage in examined brain tissue. Moreover, these characteristics do not change with rising age. Taken together, these facts show that brains of mice with altered mitochondrial function age normally. Obviously, no alteration of mitochondria and their function examined within this investigation influences the aging of healthy brains.

In the following, it will be discussed whether the combination of genetic modifications in mitochondrial DNA and AD pathology change these characteristics. Therefore, non- and APP-transgenic mice with altered mitochondrial functions were compared. This comparison revealed that all APP-transgenic chimeric mouse strains (B6mtFVB, B6mtAKR, B6mtNOD) and mouse strain APP-B6Ucp2ko have higher levels of astrocyte coverage than their non-transgenic mouse strains at

the age of 200 days (Figure 8). Obviously, increased astrocyte coverage is associated to progressing AD pathology combined with altered mitochondrial function. Nevertheless, this investigation cannot explain the association between these two factors. In contrast, only the microglia coverage of APP-B6Ucp2ko mice is upregulated compared to B6Ucp2ko mice (Figure 7). Beside KC/GRO, all other cytokines /chemokine levels are elevated in APP-B6Ucp2ko mice compared to their non-transgenic equivalent (Figure 31). Together, these data indicate that immune response to AD is altered only by Ucp2ko, a knockout in nuclear DNA. Further, it can be assumed that the enhanced astrocyte coverage is not associated with a modified immune response.

A comparison of APP-transgenic B6mtFVB, B6mtAKR, B6mtNOD and B6Ucp2ko mice to APP-B6 mice was done to assess the effects of their genetic modifications on AD. Microglia and astrocyte coverage are similar in all mouse strains at all ages. Interestingly, buffer soluble Aβ42 level of 200 days old APP-B6mtAKR mice is significantly lower than in all other APP-B6 mice with altered mitochondrial function (Figure 26). Comparing the different mutations of APP-transgenic mtDNA strains it can be hypothesized that the absence of mutations in both genes, mt-Atp8 and mt-Cox3, might lead to this decreased level. So far, little is known about this mouse strain and its characteristics. Unfortunately, this investigation did not lead to findings which point to the reason of this decrease. Moreover, data published by Scheffler et al. show that Aβ42 levels of mouse strains APP-B6mtFVB, APP-B6mtAKR, APP-B6mtNOD and APP-B6Ucp2ko are significantly lower than in mouse strain APP-B6. The reason for described differences between my investigation and Scheffler et al. was discussed in a previous section. Accordingly, further analyses to characterize APP-B6mtAKR mice are necessary.

Additionally, plaque development is not affected by mtDNA mutations, as plaque coverage, plaque size and plaque number of all, small, medium, and large plaques do not change neither at 100 nor 200 days. The only exception is that plaque size in 200 days old APP-B6Ucp2ko mice is significantly lower than in APP-B6 mice (Figure 9). Having a closer look at the consequences of Ucp2ko, it can be assumed that reduced mitochondrial membrane potential, which is a concomitant of the Ucp2ko, does not inhibit the formation of new plaques but their growth. Of course, a strong hypothesis to explain the inhibition of plaque growth would be that the phagocytosis of Aβ by microglia is increased. Unfortunately, no data regarding the phagocytosis of Aβ42 by Ucp2ko microglia have been published. However, as microglia coverage does not increase and Aβ42 level does not decrease in whole brain homogenates of APP-B6Ucp2ko mice, Aβ42 is not degraded. Moreover, astrocyte coverage between APP-transgenic B6 mice with altered mitochondrial function does not change. Therefore, further investigations to explain inhibited plaque growth in APP-B6Ucp2ko are necessary.

Within the comparison of mouse strains APP-B6, APP-B6mtFVB, APP-B6mtAKR, APP-B6mtNOD and APP-B6Ucp2ko, the significantly lower plaque size as well as the activation of immune system by Aβ42 are unique for APP-B6Ucp2ko mice. Cytokine/chemokine levels of any other mouse strain with altered mitochondrial function caused by a mutation in mtDNA (mtFVB, mtAKR, mtNOD) do not depend on the presence of APP-transgene. However, in APP-B6Ucp2ko mice the activation of immune system by Aβ42 is stronger than in APP-B6 mice, as the levels of IL-2, IL-4, IL-5, IL-6, IL-10, and IL-12p70 are increased in hemispheres of APP-B6Ucp2ko mice compared to APP-B6 mice. Some cytokine/chemokine levels are also significantly higher than in APP-transgenic B6 mice with altered mitochondrial DNA (APP-B6mtFVB, APP-B6mtAKR, APP-B6mtNOD). As mentioned before, the network of cytokines/chemokines is very complex. It is not only because of the two T cell subsets, Th1 and Th2, but also because secreting cells and target cells of these cytokines/chemokines are

not the same. So, the increased immune response is a complex that cannot be described in detail without further investigations.

Beside examining the effects of altered mitochondrial function on AD, it is important to determine whether and how they are age-dependent, especially as AD is a disease with progressing accumulation of Aβ. Astrocyte coverages of APP-B6mtAKR and APP-B6Ucp2ko rise significantly with increasing age, while microglia coverage does not change in any mouse strain with modified mitochondrial function. The levels of IL-1β, IL-4, IL-5, IL-6, IL-10 and IL-12p70 are significantly higher in APP-B6Ucp2ko compared to APP-B6 mice. Taken together, these data lead to the hypothesis that immune response rises with increasing age in APP-B6Ucp2ko mice only.

The results of APP-B6mtFVB mice have been discussed in previous sections especially regarding plaque coverage, plaque number and the number of small, medium, and large plaques with rising age. Interestingly, Aβ42 levels follow the same pattern. Buffer and guanidine soluble Aβ42 levels of APP-B6mtFVB mice increase significantly from 100 to 200 days. In 100 days old APP-B6mtAKR mice buffer soluble Aβ42 level is significantly lower than in all other mouse strains with altered mitochondrial functions. Here Aβ42 is undetectable. This might have been caused by a dilution which was too high. A second measurement of these samples was not possible to repeat it with lower dilutions due to sample volume limitations. Plaque formation is not affected by any other mouse strain with a variation in mitochondrial or nuclear DNA. Taken together, these data indicate that APP-B6Ucp2ko is the only mouse strain with an upregulation of immune response with rising age. As this increased immune response is age-related in APP-B6Ucp2ko but not B6Ucp2ko mice, Aβ is causing it. However, the characteristics of AD progression in APP-B6Ucp2ko mice are not reflected by significant changes when compared to APP-B6 mice within this investigation.

To sum up, except altered mitochondrial functions caused by the Ucp2ko or mtFVB specific SNPs no other mitochondrial DNA alteration showed significant influence on aging of healthy brains or AD pathology. So, chimerism does not affect AD pathology in general. This fact is very surprising as the mitochondrial cascade hypothesis, which is broadly accepted, describes the link between oxidative stress (reactive oxygen species etc.) and AD and it is supported by numerous findings (reviewed in [134]). Only the combination of mtDNA of FVB mice in B6 background has a strong influence and it was already discussed in previous sections. On the one hand, this mutation enhances AD pathology far more than Ucp2ko. On the other hand, immune response to AD is stronger in B6 mice with Ucp2ko than with mtDNA of FVB mice. So, decreased membrane potential caused by Ucp2ko affects cytokine/chemokine levels and microglia coverage in 200 days old APP-B6Ucp2ko, while altered ATP synthase in APP-B6mtFVB mice predominantly influences plaque development.

A strong correlation between ABC transporter function and AD pathology was described in previous studies [50,52,135]. Fortunately, ABC transporters are well-characterized as they are an attractive drug target for chemotherapy. While a down-regulation of their activity increases efficiency of cancer treatment, previous studies revealed that a decreased AD pathology is related to increased activity of ABC transporters. Therefore, it is of high interest to investigate the complex relations between ABC transporters, immune response and AD.

So far, all investigations dealing with the influence of ABC transporter knockouts have been performed with mice in FVB background. As demonstrated in this and previous studies, B6 and FVB mice respond different to AD. Therefore, it is important to examine whether genetic background interferes with the effects of ABC transporter knockouts. ABC transporter knockouts in FVB background do not alter Aβ40 level, astrocyte coverage and microglia coverage of non-transgenic

FVB mice. Furthermore, these characteristics are not age-dependent. Out of all examined cytokine levels only IL-4 level of 200 days old FVB-B1 mice is increased in comparison to 200 days old FVB mice and 100 days old FVB-B1 mice (Figure 33). Additionally, this level is significantly higher than in 200 days old FVB-G2 mice. These results are surprising as previous research revealed that ABCC1 transports IL-4 whereas no indications exist for ABCB1 [136]. Probably, FVB-B1 mice have increased numbers of Th2 cells in their brains, as IL-4 induces differentiation of Th0 cells to Th2 cells. Related to this hypothesis it can be assumed that FVB-B1 mice respond Th2 prone. The pathway of IL-4 is complex and cannot be explained in detail without further examinations.

The data of non-transgenic FVB mice with ABC transporter knockouts described in the previous passage help to interpret data measured in APP-FVB mice with ABC transporter knockouts (ABCB1, ABCC1, ABCG2). APP-FVB-B1, APP-FVB-C1 and APP-FVB-G2 were investigated at both ages, 100 and 200 days. A comparison of non- and APP-transgenic ABC transporter knockout mice revealed a few significant differences at the age of 200 days. 100 days old APP-FVB-B1, APP-FVB-C1 and APP-FVB-G2 mice do not differ in the examined characteristics compared to their non-transgenic equivalents. At 200 days, only ABCC1 knockout mice show differences between non- and APP-transgenic mice with FVB background. Astrocyte coverage and IL-1β level are significantly higher in APP-FVB-C1 mice than in FVB-C1 mice (Figure 13). The combination of these data with previously discussed data shows that increased astrocyte coverage is caused purely by ABCC1 knockout combined with progressing Aβ accumulation. The enhanced astrocyte coverage also occurs in B6 background.

A comparison of APP-FVB, APP-FVB-B1, APP-FVB-C1 and APP-FVB-G2 revealed that these strains do not differ significantly in microglia coverage, plaque formation or levels of buffer and guanidine soluble Aβ42. Astrocyte coverage is significantly increased in 200 days APP-FVB-C1 mice in comparison to 200 days old APP-FVB, APP-FVB-B1 and APP-FVB-G2 mice. This effect of ABCC1 knockout is accompanied by downregulated levels of IL-2, IL-6 and KC/GRO at 100 days. These downregulations are not apparent anymore at 200 days.

None of the examined ABC transporter knockouts has an influence on plaque formation as well as buffer and guanidine soluble Aβ42 levels at 100 or 200 days. Nevertheless, these ABC transporters affect some cytokine/chemokine levels. For example, the level of KC/GRO is significantly decreased in 100 days old APP-FVB-B1, APP-FVB-C1 and APP-FVB-G2 mice compared to 100 days old APP-FVB mice. At 200 days, the KC/GRO levels are similar again. KC/GRO is secreted by cells forming the blood-brain barrier, astrocytes, and endothelial cells. Vukic et al. found that cultured human brain endothelial cells exposed to Aβ have increased gene expression of KC/GRO among others [137]. Furthermore, Zhang et al. showed that KC/GRO contributes to a migration of monocytes from blood to brain which is induced by Aβ [138]. Taken together, these facts indicate that knockouts of ABCB1, ABCC1 and ABCG2 contribute to the production of KC/GRO in the presence of Aβ. This effect is not specific for one ABC transporter even though the investigated ABC transporters have different characteristics and functionalities. Therefore, it is likely that these differences are indirect effects. Moreover, it can be assumed that the KC/GRO level also depends on another factor as the ABC transporter knockouts do not lead to decreased KC/GRO levels at 200 days anymore.

Additionally, IL-2 level is significantly higher 100 days old APP-FVB mice than in APP-FVB-B1, APP-FVB-C1 and APP-FVB-G2 mice. IL-6 level is significantly lower in 100 days old APP-FVB-C1 mice than in APP-FVB mice. Interestingly, both differences were not observed at 200 days anymore. Although data published in Alves et al. suggest that IL-2 weakens AD pathology, results of this investigation do not support this hypothesis. Furthermore, Cojocaru et al. revealed that IL-6 level is increased in AD patients [139]. These data prove the connection between Alzheimer's disease and IL-2 as well as

IL-6 but so far, no data linking these cytokines to ABC transporters ABCB1 and ABCC1 exist. Additionally, the IL-1β level of mouse strain APP-FVB-G2 is significantly reduced in comparison to mouse strains APP-FVB, APP-FVB-B1, and APP-FVB-C1 at 200 days. As mentioned before, the role of IL-1β in AD is well known. For example, Zhang et al. showed in *in vitro* experiments that exposure to Aβ leads to increased levels of IL-1 β and IL-6 [140]. Contrary, the link between these interleukins and ABC transporters is not well examined. Interestingly, Cartwright et al. already proposed a regulation of transport by ABCB1 and ABCC1 that includes TNFα [141].

Beside the effects of ABCB1, ABCC1 and ABCG2 knockouts at 100 and 200 days, it is important to analyse the effect of increasing age on each of these knockouts. Astrocyte coverage increases between 100 and 200 days only in APP-FVB-C1 mice. This enhancement does not affect plaque number, plaque coverage or any other characteristic of plaque formation. Interestingly, the IL-1β level of APP-FVB-C1 mice increases significantly with rising age. Overall, these findings once more highlight the combined effect of APP-transgene and age on IL-1β level. Even though the TNFα level of APP-FVB mice is significantly different than some other mouse strains, it should be taken into consideration that the measurements of cytokine/chemokine levels of 200 days old APP-FVB mice are based on only two mice. Therefore, the reliability of these results is limited. Still, these findings show that ABCC1 is an outstanding ABC transporter which affects AD pathology and its accompanied immune response more than ABCB1 and ABCG2.

Furthermore, ABC transporter knockout mice (ABCB1, ABCC1, ABCG2) were backcrossed to B6 background and compared to each other. This comparison shows that microglia coverage, astrocyte coverage, Aβ40 levels and cytokine/chemokine levels are not affected by ABC transporters neither at 100 days, 200 days or with rising age. These results show that ABC transporter knockouts do not affect these characteristics no matter whether these knockouts were in FVB or B6 background. One exception is that the IL-4 level of B6-B1 mice decreases with rising age (Figure 12). Interestingly, the level of IL-4 is influenced by ABCB1 knockout in genetic backgrounds FVB as well as B6. It decreases significantly with rising age in B6-B1 mice. This progression is contrary to FVB background. To sum up, ABCB1 knockout affects IL-4 level and the genetic background strongly influences the direction of this effect.

Based on control data of non-transgenic mice, ABC transporter knockouts in APP-transgenic B6 mice were examined and compared to their non-transgenic equivalent. Due to the presence of Aβ plaques, a significant increase of cortical coverage with glial cells was expected. Unfortunately, no data of 200 days old APP-B6-B1 and APP-B6-G2 mice exist due to sample limitations. In 200 days old APP-B6-C1 mice a rise of coverage with glial cells was proven in comparison to their non-transgenic equivalents (Figure 10, Figure 12). However, cytokine/chemokine levels are not significantly altered by an ABCC1 knockout no matter whether mice are non- or APP-transgenic. These results indicate that the APP-transgene has no effect on immune response in any B6 mice with ABC transporter knockout. 200 days old APP-B6-B1 and APP-B6-G2 mice also need to be analysed to confirm this hypothesis and to test whether they lead to an earlier increase in immune response to AD.

The next step of this investigation is a comparison of APP-B6 and APP-B6-C1 mice. Due to the mentioned sample limitation, it is not possible to include mouse strains APP-B6-B1 and APP-B6-G2 in this comparison. At 100 and 200 days, microglia coverage, astrocyte coverage, plaque formation (plaque number, plaque size etc.), Aβ42 level and cytokine/chemokine levels do not differ between APP-B6 and APP-B6-C1 mice. The only exception is that microglia coverage and astrocyte coverage are altered by ABCC1 knockout. Indeed, the coverage of cortical area with glial cells of 200 days old APP-B6-C1 mice is significantly increased in comparison to 200 days old B6-C1 mice, 100 days old

APP-B6-C1 mice and 200 days old APP-B6 mice. Therefore, it can be assumed that ABCC1 knockout is an enhancer for AD pathology and its associated immune response. No other examined characteristic is changed by ABCC1 knockout in B6 background, independent of age and the presence of APP-transgene.

To conclude, no examined ABC transporter affects the progression of AD pathology neither in B6 nor in FVB background. Nevertheless, significant changes of astrocyte coverage caused by ABCC1 knockout are independent of nuclear DNA as they appear in B6 and FVB background. In marked contrast to immunohistochemical analyses, quantifications of cytokine/chemokine levels revealed a few differences between effects of ABC transporter knockouts in B6 and FVB background.

Finally, this investigation checked whether the effects of ABCB1, ABCC1 and ABCG2 significantly depend on nuclear background. The first step to check this is a comparison of non-transgenic B6 and FVB mice with knockouts of ABCB1, ABCC1 and ABCG2. This comparison revealed that Aβ40 level of 100 days old FVB-G2 mice it is significantly higher than in 100 days old B6-G2 mice, while all other ABC transporter knockouts have no effect. This change of Aβ40 level is accompanied by increased IL-5 level in FVB background. Furthermore, IL-4 level is enhanced in 200 days old FVB-B1 mice compared to 200 days B6-B1 mice. All other characteristics are similar in non-transgenic mice with ABC transporter knockouts in B6 and FVB background. However, the significant changes of Aβ40 levels and IL-4 levels are minor ones in a complex system that do not show a pattern. Therefore, further investigations are necessary to assess the importance of these significant differences.

A comparison of APP-transgenic mice with ABC transporter knockouts in B6 and FVB background showed similar diffuse results. Between these strains neither plaque number, astrocyte coverage nor cytokine/chemokine levels differ. Indeed, ABCC1 knockout leads to an increased microglia coverage in 200 days old APP-B6-C1 mice compared to its FVB background counterpart. In 100 days old APP-B6-B1 mice, guanidine soluble Aβ42 level of APP-FVB-1 is significantly increased compared to their equivalent mice in FVB background. Additionally, cytokine/chemokine levels in ABC transporter knockout strains are not different between nuclear backgrounds B6 and FVB. All these results do not show any recurring pattern. Therefore, they cannot be explained without further investigations. Nevertheless, these results are important as they show once more that genetic backgrounds affect a broad range of characteristics. Moreover, it is important to take them into consideration in future experiments.

This new insight is highly interesting for every experiment using these mouse models to investigate the effects of ABC transporters on AD pathology. Furthermore, these data raise the question whether results gained by Krohn et al. can be reproduced with mice in B6 background [52]. If treatment with thiethylperazine in APP-B6 mice reduces Aβ42 level and plaque numbers as efficient as in APP-FVB mice, as described in Krohn et al., it would support the hypothesis that the effect mechanism of thiethylperazine is not only specific for ABCC1 but also independent of genetic background. This would be highly beneficial as a treatment success in mouse models that does not depend on nuclear background is more promising to be effective in all patients.

The purpose of this investigation was to check the hypothesis that AD is a multifactorial disease influenced by nuclear and mitochondrial DNA, ABC transporters and immune response. Summarizing all data, AD is clearly a disease indirectly affected by all mentioned factors. For example, in contrast to ABCG2, ABCB1 affects IL-4 levels and responses whereas an ABCC1 knockout alters astrocyte coverage. Regarding mitochondrial alterations, it can be summarized that in the presence of Aβ all kinds of chimerism lead to increased astrocyte coverage. Furthermore, the

chimerism of B6mtFVB mice and Ucp2ko have the strongest influence on AD pathology. Even though their age-related consequences are different. While both types of chimerism increase buffer soluble Aβ42 level and astrocyte coverage, other characteristics of plaque development are distinct between these altered mitochondrial functions. The combination of mtDNA of FVB mice and nuclear DNA of B6 mice leads to stronger age-related increase of guanidine soluble Aβ42 levels associated to enhanced formation of new plaques. Contrary, Ucp2ko affects microglia coverage and cytokine/chemokine levels but not plaque development.

Regarding immune response, it can be summarized that each analysed cytokine/chemokine has an influence on AD pathology. Nevertheless, all cytokine/chemokine levels depend on other factors like background, mitochondrial mutations, or ABC transporters. This investigation shows that FVB background supports stronger immune responses than B6 background and it proves that the influence of immune response on AD cannot be conclusively estimated and should be examined with further measurements. Especially, as the data presented are not sufficient to explain effects on signalling pathways of cytokines and chemokines.

However, although all data were obtained with reliable methods, their difference to previous publications cannot be explained so far. Therefore, further experiments are necessary to check the cause or to confirm the results of immunohistochemistry and protein biochemistry as these data are important for future experiments.

6. Outlook

An important subpart of this study has been an extensive and comparative characterization of background strains B6 and FVB. Further comparative measurements of cytokine/chemokine levels should extend this characterization. Of note, comparative characterizations are rare but highly helpful. For example, it can be examined which background strain simulates conditions in humans more proper. In future, these data will help to optimize circumstances of treatment studies in mouse models that shall be applied to humans later. In general, characteristics of the examined mouse strains can be used to determine which mouse model is the best choice for future experiments.

Additionally, further investigations of APP-B6Ucp2ko and APP-B6mtFVB mice should be performed to find out why and how chimerism of B6mtFVB mice and Ucp2ko affect AD pathology via immune response. Obviously, these altered mitochondrial functions have effects on plaque development that might be beneficial. Disclosing this secret could be helpful for conceiving further insight in the formation of plaques. One focus of these investigations should be the possible links to IL-1β and IL-10.

Even though results of this examination did not reproduce previously published data, the role of ABC transporters is not raised to question. Therefore, additional research on APP-transgenic mice with ABCC1 knockout will help to understand the transport of Aβ through blood-brain barrier. This investigation once more showed that ABCC1 has stronger effects i.e. on astrocyte coverage in comparison to ABCB1 and ABCG2s. Therefore, this ABC transporter is the best one to check the effect of inhibited or enhanced Aβ transport via the blood-brain barrier and on the progress of AD pathology, especially the accumulation of Aβ. In further experiment with this subject it is advisable to check the role of cytokines and chemokines. Especially, if these experiments are performed in FVB background.

So far, all factors like genetic background, altered mitochondrial function, neuroinflammation and ABC transporters have been investigated separately. In this investigation, also the links between these factors were examined for the first time. Results of these examinations show that they should be extended. Especially, an examination of the relation between ABC transporter activity and altered mitochondrial function should be performed, as the activity of ABC transporters highly depends on the availability of ATP.

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Appendix

Exemplary immunohistochemical stainings of non-transgenic mouse strains

In the following, figures show stainings exemplary for the quantifications of astrocyte (GFAP) and microglia (IBA1) coverage in non-transgenic mice. A panel of four figures consists of GFAP (left) and IBA1 (right) stainings of 100 (top) and 200 (bottom) days old mice. For mouse strain B6-B1 only a group of 200 days old mice was available for stainings.
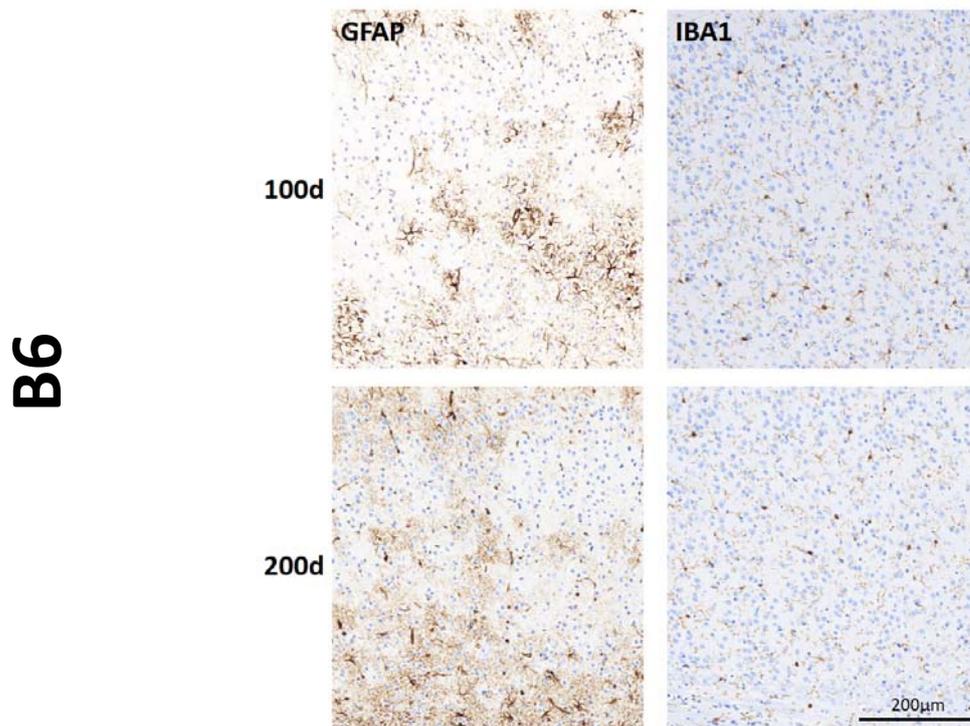
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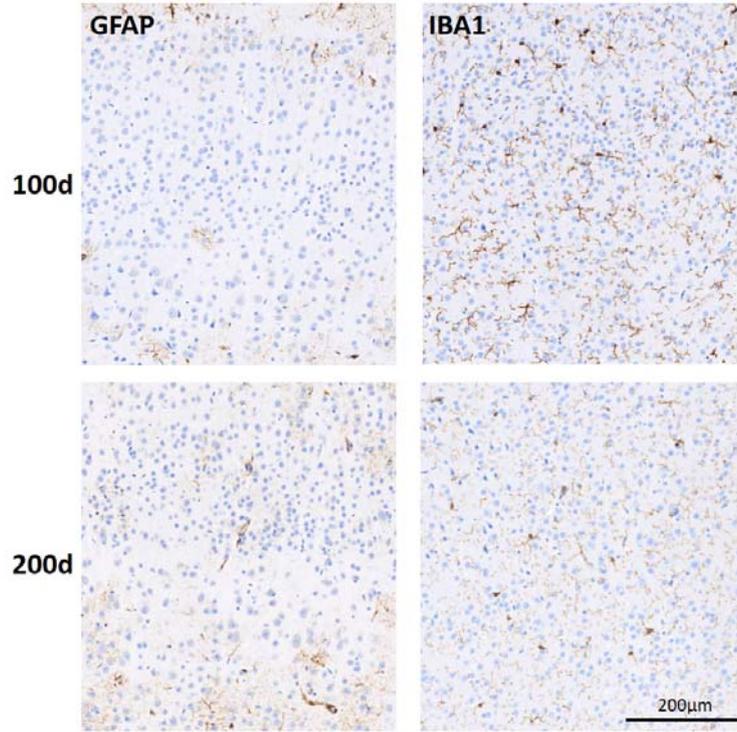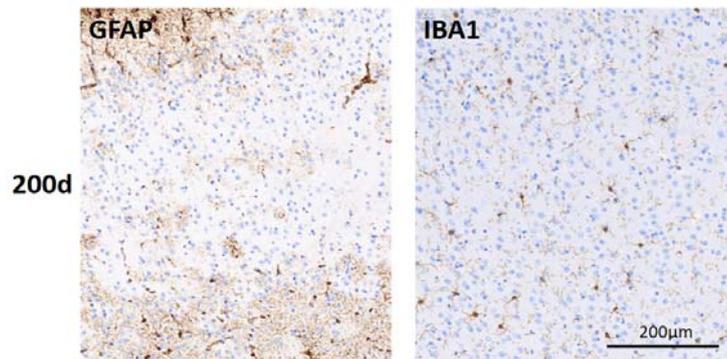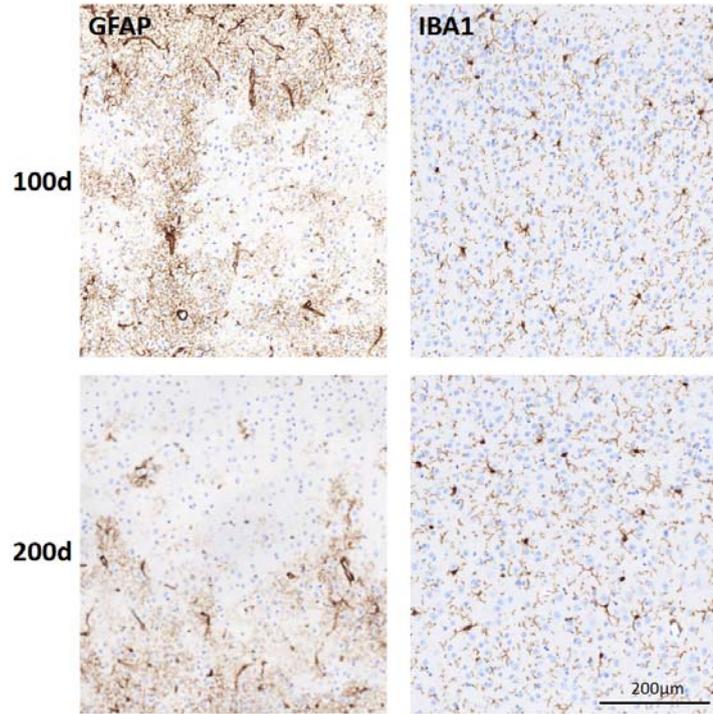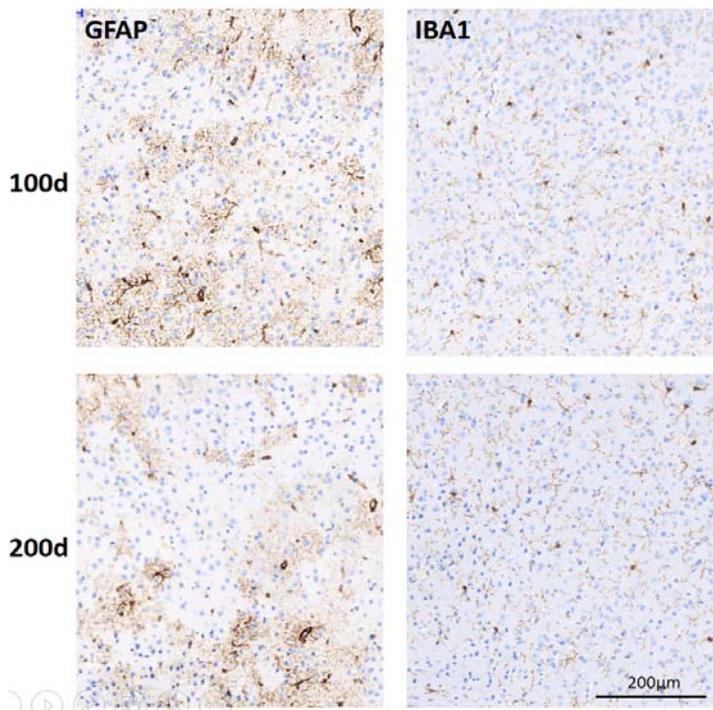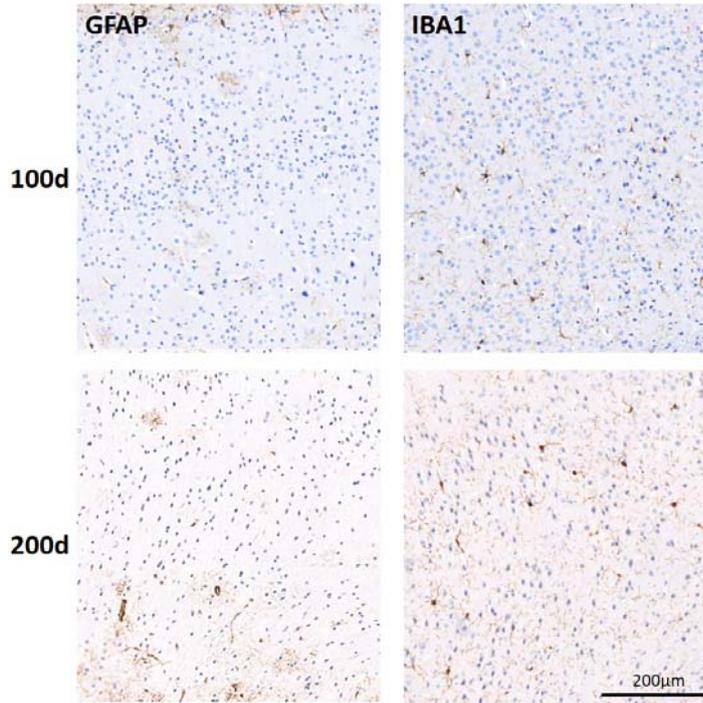
GFAP IBA1

100d

200d

200μm

B6-B1

GFAP IBA1

200d

200μm

B6-C1

GFAP   IBA1

100d

200d

200μm

B6-G2

GFAP   IBA1

100d

200d

200μm

FVB-G2

B6mtFVB

GFAP IBA1

100d

200d

200μm

GFAP IBA1

100d

200d

200μm
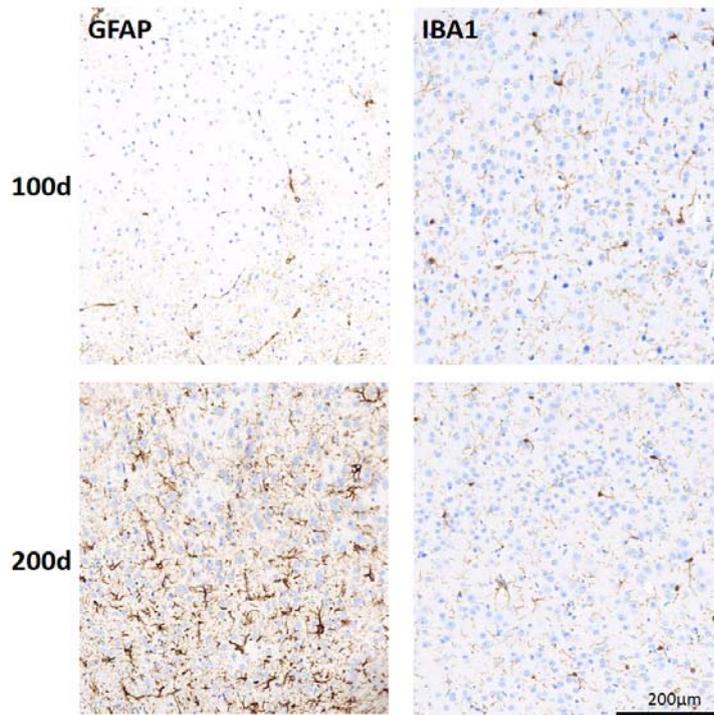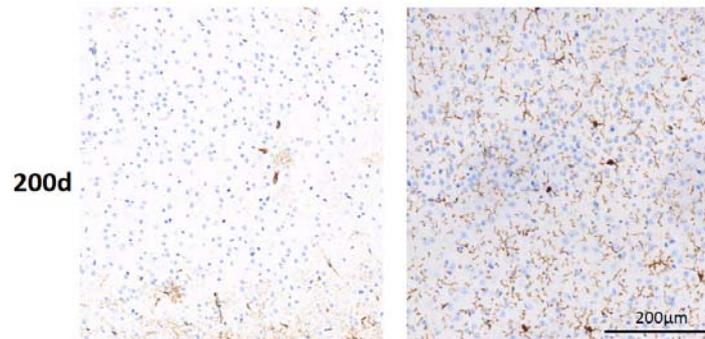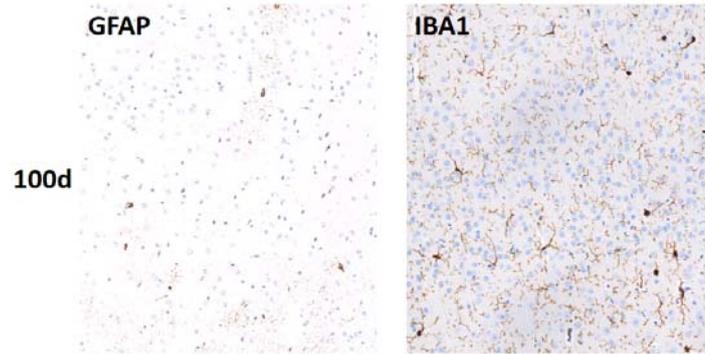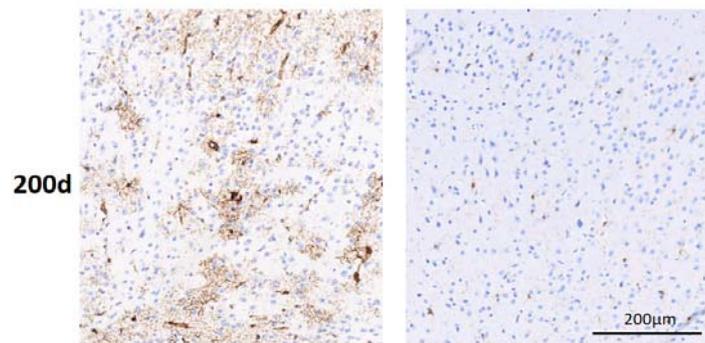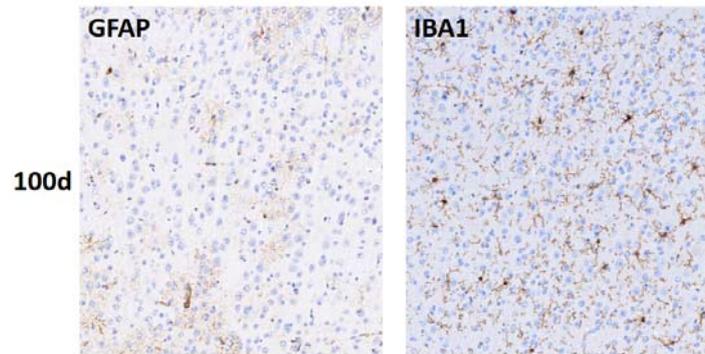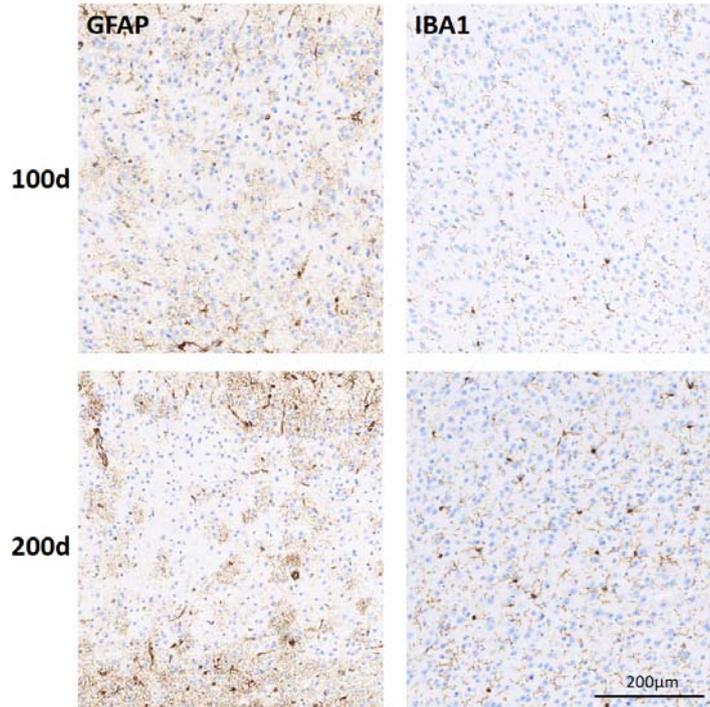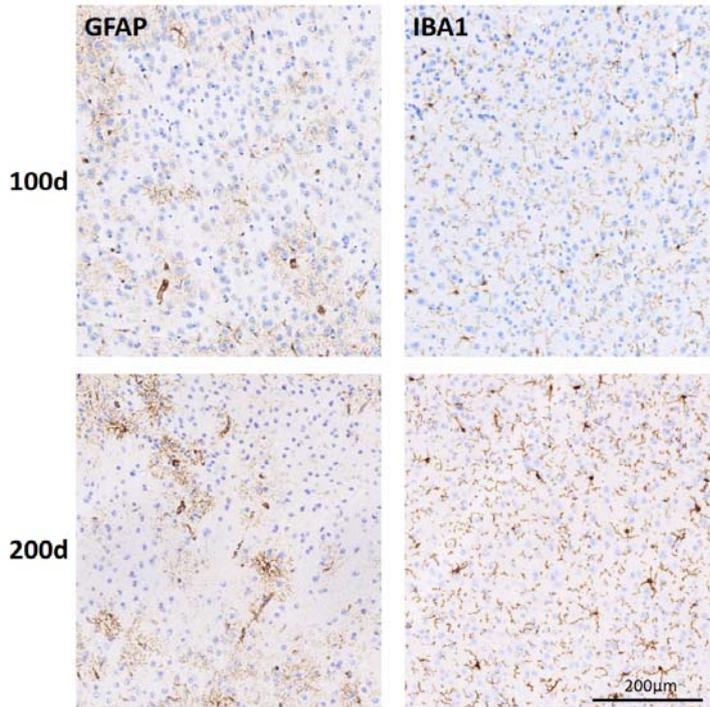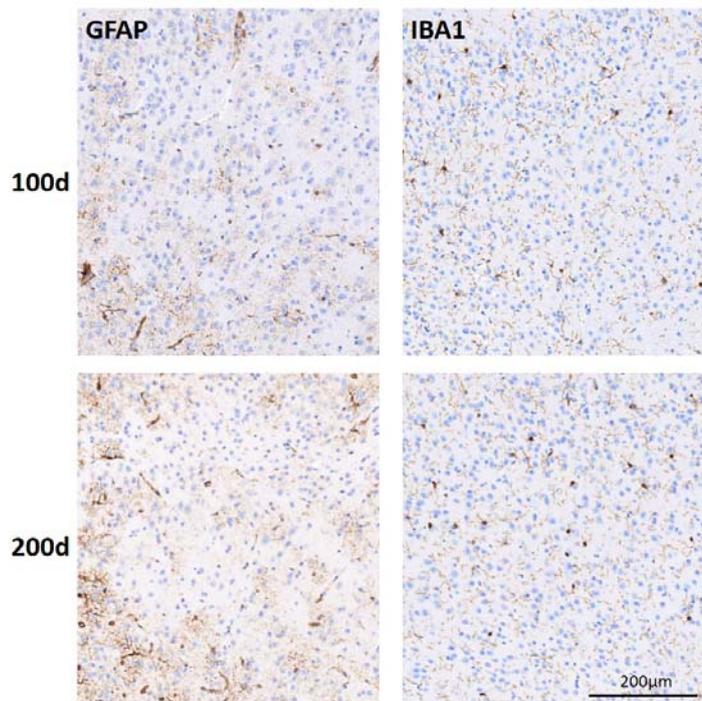
Exemplary immunohistochemical stainings of APP-transgenic mouse strains

In the following, figures show exemplary stainings for the quantifications of astrocyte coverage (GFAP), microglia (IBA1) and plaque development in APP-transgenic mice. A panel of six figures consists of GFAP (left), IBA1 (middle) and 6F3D (right) stainings of 100 (top) and 200 (bottom) days old mice. Due to sample limitations, it was not possible to stain 200 days old APP-B6-B1 mice or APP-B6-G2 mice (100 and 200 days).

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Declaration/ Erklärung

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Hiermit erkläre ich, dass ich die von mir eingereichte Dissertation zu dem Thema

„The role of mitochondrial function and ABC transporters in healthy aging and Alzheimer's disease in the light of genetic modifications"

selbstständig verfasst, nicht schon als Dissertation verwendet habe und die benutzten Hilfsmittel und Quellen vollständig angegeben wurden.

Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.) an anderen Einrichtungen eingereicht habe.

Magdeburg, 13.04.2017

Curriculum vitae

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	Diploma thesis at the Neurodegeneration Research Laboratory
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1997 – 2005	Ernst-Barlach-Gymnasium, Rostock, Germany, A-levels

List of publications

Steffen J, Krohn M, **Paarmann K**, Schwitlick C, Brüning T, Marreiros r, Müller-Schiffmann A, Korth C, Braun K, Pahnke J (2016) – *Revisting rodent models: Octodon degus as Alzheimer´s disease model?* Acta Neuropath Com

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