Lipocalin 2 and the pathophysiology of Alzheimer's disease
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2019

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Chapter 7

General discussion
General discussion

Alzheimer’s disease (AD) is a devastating disease, for which currently no efficient curative treatments exist. The AD brain is characterized by different pathological changes, including abnormal aggregation of amyloid-β (Aβ) and tau proteins, chronic neuroinflammation (mediated by chronically activated microglia and astrocytes) and disturbed iron metabolism [1–3]. However, the exact mechanisms that drive the development of these pathological processes, and the mechanisms that underlie the toxic effects of these pathological processes, are not completely understood yet. Therefore, tremendous efforts are invested to discover novel mechanisms and factors that may be important in the development and progression of AD. These studies will help to unravel the pathological processes that underlie AD, and may identify new therapeutic targets to treat AD. It is becoming increasingly clear that chronic (neuro)inflammation plays a key role in the pathophysiology of AD [2]. As such, better insights into the neuroinflammatory mechanisms and factors involved in AD may be crucial to understand and treat AD.

About 7 years ago, the protein Lipocalin 2 (Lcn2) was found to be a potential important new inflammatory player in AD [4]. Lcn2 (also known as neutrophil gelatinase-associated lipocalin (NGAL) in humans) has antibacterial functions, and is also involved in inflammatory processes, iron metabolism and cell death/survival signaling [5,6]. Lcn2 expression in the periphery and the brain is low under healthy conditions. Interestingly, Lcn2 levels were found to be increased in the brain in different brain diseases, such as Parkinson’s disease (PD), Multiple Sclerosis and stroke. Although some contradictory findings have been reported, most studies in animal models of these brain diseases indicated that Lcn2 could significantly exacerbate brain pathology, by for example promoting neuroinflammation, iron accumulation and cell death [6,7]. Regarding AD, it was shown that Lcn2 levels are significantly increased in human brain regions affected by AD pathology [4]. Moreover, studies in cultured brain cells showed that Lcn2 expression is strongly induced in astrocytes upon Aβ exposure, and that Lcn2 aggravates Aβ-induced cell death [4,8]. In addition, Lcn2 expression in the periphery (and the brain, as was shown in some cases) was found to be increased during different risk factor conditions of AD, including increasing age, obesity, physical inactivity and depression [9–14]. This indicates that Lcn2 might affect the brain already during early stages of AD. Collectively, these findings suggest that Lcn2 might be involved in the development and progression of AD. However, more work – including investigations in animal models of AD – is required to elucidate the effects and importance of Lcn2 in the pathophysiology of AD. Therefore, in this thesis we aimed to further explore the role of Lcn2 in AD, by studying this protein in human AD tissues and in mouse and cell culture models of AD. In addition, the work presented in this thesis provides further insights in the characteristics of Lcn2 as a potential diagnostic marker and therapeutic target for AD.
Summary of main findings

In summary (Fig. 1), in chapter 2 of this thesis we first confirmed that Lcn2 protein levels are increased in human post-mortem brain tissue of AD patients, as compared to healthy age-matched controls. Besides increased Lcn2 levels in the hippocampus as was shown before [4], we also found increased Lcn2 levels in other brain regions that are affected by AD, including the prefrontal cortex. In addition, we observed that Lcn2 levels significantly differ between AD patients with and without co-existing depression, in different brain regions. Consistent with previous results, Lcn2 levels in blood were similar in AD and control patients, and Lcn2 levels in the cerebrospinal fluid (CSF) were decreased in AD patients as compared to healthy controls [4,15]. Considering the increased Lcn2 levels found in the human AD brain, in chapter 3 we aimed to confirm that Lcn2 levels are also increased in a mouse model of AD (the J20 mouse model). Moreover, we aimed to explore whether Lcn2 may significantly contribute to AD-like pathology in this AD mouse model. The results from this study confirmed that also in a mouse model of AD, brain Lcn2 levels are increased. Notably, when we compared AD mice and Lcn2-deficient AD mice at 12 months of age, we found no differences in the severity of many AD-like characteristics, including cognitive impairment, Aβ plaque load, and activation of microglia and astrocytes. However, we did find more severe iron accumulation in the brains of AD mice as compared to Lcn2-deficient AD mice. In chapter 4, we aimed to confirm a previous finding showing that Aβ provokes Lcn2 production in astrocytes [8]. Moreover, considering the potential value of Lcn2 as a therapeutic target for AD and indications that iron chelators may reduce Lcn2 overexpression in the injured brain [16,17], we aimed to explore whether iron chelators may be able to decrease Aβ-induced Lcn2 overproduction. Indeed, experiments in cultured astrocytes confirmed that Aβ
exposure induces the production of Lcn2, and that this Aβ-induced Lcn2 expression could be blocked by iron chelators. Additionally, results suggested that Aβ may induce iron accumulation in astrocytes, and that Lcn2 does not significantly affect this Aβ-induced change in astrocytic iron metabolism. In chapter 5, we aimed to gain more insight into the characteristics of Lcn2 as a potential diagnostic marker. The findings from this study indicated that Lcn2 levels in blood and CSF do not display significant circadian fluctuations, in healthy elderly males. The circadian stability of Lcn2 may be beneficial, when Lcn2 would be used as a biomarker. Finally, in chapter 6, we aimed to summarize the current evidence for a potential role of Lcn2 in different age-related brain diseases, including AD, PD and vascular dementia (VaD). In addition, we discussed the involvement of Lcn2 in conditions that increase the risk to develop these brain diseases, and explored the possibility that Lcn2 is a biological link between risk factor conditions and the development of AD, PD and VaD.

Overall, these findings suggest that AD(-like) pathology in humans and mice is consistently accompanied by increased Lcn2 levels in the brain, which may – at least when studied in a 12-month old J20 mouse model of AD – modestly contribute to certain aspects of AD pathology. However, it is possible that stronger effects of Lcn2 may present themselves in other (e.g. more severe) animal models of AD and/or at other ages. As such, more research is required to obtain a better understanding of the effects of Lcn2 in AD. A more thorough insight into the importance and mechanisms of action of Lcn2 in AD will also clarify the potential promise of Lcn2 as a therapeutic target and diagnostic marker for AD.

Although new insights were obtained, the results in this thesis also point out important gaps in the knowledge regarding Lcn2’s functions in the healthy and AD brain. As such, in the following paragraphs we will discuss and propose what investigations are necessary to carry out next, and what drawbacks and opportunities should be looked out for, in future investigations of Lcn2 and AD.

The regulation of Lcn2 expression and sources of Lcn2 in AD

We detected increased Lcn2 protein levels in human AD brain tissue as well as in brain tissue of J20 AD mice, as compared to healthy control tissue (chapter 2 and 3). These findings are in line with the previously reported elevated hippocampal levels of Lcn2 in human AD brain tissue [4]. Similarly, increased mRNA expression levels of Lcn2 were previously found in the hippocampus and choroid plexus of APP/PS1 AD mice and WT mice after an intracerebroventricular injection with oligomeric Aβ, as well as in the brain of Tg2576/PS-1P264L/P264L AD mice [18,19]. In addition, we found that treatment with Aβ1-42 significantly induces the production of Lcn2 in cultured primary astrocytes (chapter 4), which corresponds with a previous study [8]. The signaling pathways that are involved in AD-related upregulation of Lcn2 remain to be explored further, but appear to at least include activation of tumor necrosis factor receptor 1 (TNFR1) [4,18]. Also, iron may be involved in the Aβ-mediated induction of Lcn2 (although iron alone does not seem sufficient to induce Lcn2 production in primary cultured astrocytes, chapter 4) [16,17]. As such, blocking overactive TNFR1 signaling and reducing iron accumulation in the AD brain may decrease/normalize
Lcn2 expression. Both TNFR1 antagonists and iron chelators have been shown to exert neuroprotective effects in animal models of different brain diseases, including AD [3,18,20]. It would be of interest to establish to what extent these neuroprotective effects may rely on the reduction of Lcn2 overexpression. Moreover, developing therapies that target Lcn2 expression or Lcn2 activity specifically might be of interest as well, when enough evidence indicates that reducing Lcn2 overexpression would be an effective and safe therapeutic strategy in AD.

The cellular sources of the increased Lcn2 levels in AD require further study as well. In hippocampal brain sections of both human AD patients and J20 AD mice, we observed that Lcn2 is mostly localized in astrocytes, and not in neurons and microglia (chapter 2 and 3). This suggests that astrocytes may be the main source of Lcn2 in the brain (although we did not study the cellular location of Lcn2 mRNA), which has been reported before [8,21–23]. Nevertheless, it is known that other cell types may be important sources of Lcn2 in the brain as well, including for example brain endothelial cells, choroid plexus epithelial cells and infiltrating neutrophils [8,24–31]. In future research, it would be of interest to also study these possible sources of Lcn2. In addition, it has been shown that Lcn2 protein in the circulation is able to pass the BBB [32]. Therefore, it would be interesting to determine to what extent peripheral Lcn2 levels may contribute to rising Lcn2 levels in the brain. Of note, it might be possible that the cellular sources of Lcn2 change with time, e.g. depending on age and the disease stage. Besides investigating the production and sources of Lcn2 once AD pathology is established, it would also be interesting to study this in risk factor conditions and early stages of AD. For example, Lcn2 levels in the brain were suggested to increase already during certain risk factors of AD, including advancing age, unhealthy diet, obesity and psychological stress [13,26,33–36]. This indicates the possibility that increasing Lcn2 levels in the brain during risk factor conditions of AD may correlate with an increased risk to develop AD (chapter 6). Depression is a risk factor to develop AD, and the co-existence of depression in AD may lead to an accelerated progression of AD pathology [37–39]. In chapter 2, we described that co-existing depression in AD patients is related with significantly higher hippocampal Lcn2 levels, as compared to AD patients that did not suffer from co-existing depression. Contrarily, in certain other brain regions, Lcn2 levels were decreased in AD patients with co-existing depression, as compared to non-depressed AD patients. These findings indicate that the regulation of Lcn2 expression is complex and may differ significantly between brain regions, depending on the presence of specific co-morbidities. Of note, besides determining the cellular sources of Lcn2, it would also be of interest to assess the cell types that take up most Lcn2 and are most sensitive towards Lcn2, which may depend on the relative expression levels of Lcn2 receptors, including 24p3R, megalin and melanocortin 4 receptor (MC4R). In addition to cell types that take up Lcn2, Lcn2 might also be present in other structures. For example, neutrophils can release neutrophil extracellular traps (NETs). NETs are networks or extracellular fibers (mainly composed of chromatin from neutrophils) decorated with antimicrobial proteins, which are able to bind and kill microbes [40,41]. NETs were also found in AD brain tissue, and were suggested to exacerbate neuroinflammatory
processes (and thereby vascular and brain damage) in AD [42]. Interestingly, Lcn2 was shown to be a component of NETs in certain peripheral diseases, and to contribute to the antimicrobial effects of NETs [42]. Perhaps, Lcn2 may also be involved in the suspected damaging effects of NETs in the AD brain. Besides NETs, it may be hypothesized that Lcn2 binds Aβ oligomers and/or plaques in the AD brain (and potentially contributes to the antibacterial effects described for Aβ [43,44]). However, this possibility requires further investigation.

The potential of Lcn2 as a biomarker for AD requires further exploration. It is important to note that increased Lcn2 levels are in general very unspecific, since Lcn2 levels are elevated in many infections, injuries and diseases [5–7]. However, it is possible that for example altered Lcn2 levels in CSF may be more specific for AD. Lcn2 levels in CSF are significantly decreased in AD patients as compared to controls, which mimics the lower CSF levels of Aβ in AD patients (chapter 2, [4,45,46]). A possible explanation for this is that the clearance of both Aβ and Lcn2 from the brain is hampered in AD, potentially due to lower blood-brain barrier expression of e.g. megalin, which may be able to transport both Aβ and Lcn2 out of the brain [4,47,48]. Interestingly, Lcn2 levels in CSF are also decreased in people with mild cognitive impairment (MCI), which is a prodromal stage and risk factor of AD [4]. Potentially, in combination with other AD-specific disease markers, reduced CSF Lcn2 might improve the early and accurate diagnosis of AD. In this context, it is of benefit that CSF Lcn2 levels appear to be stable over the day, without significant circadian fluctuations (chapter 5). However, this finding was made in healthy elderly males and as such should be confirmed in females, as well as in people with age-related central nervous system (CNS) diseases including AD. Lcn2 levels in blood serum do not differ between AD patients and healthy age-matched controls (chapter 2, [4,15]). However, it might be an interesting opportunity to assess Lcn2 levels in specific cells in the blood, such as neutrophils. Neutrophils store Lcn2, and have been shown to infiltrate the AD brain [42,49–51]. Possibly, differential levels of Lcn2 might be present in primed neutrophils, in the circulation of AD patients. In addition, it may be of interest to study Lcn2 levels in feces. Potentially, Lcn2 levels in feces may aid in discovering gut microbiome changes which are known to arise in AD [52]. Lastly, when exploring Lcn2 as a biomarker, the different states in which Lcn2 can exist should be taken into account (reviewed in chapter 6). Although their respective functions and properties are not completely clear yet, differences in for example iron-bound and iron-free, monomeric and dimeric and (non-) polyaminated states of Lcn2 might potentially relate to specific diseases.

Effects of Lcn2 in the J20 mouse model of AD
Is increased Lcn2 production in AD merely a side-effect of disease, or does it also contribute to AD pathology? This question was addressed in chapter 3 of this thesis, by comparing four groups of mice: normal wild-type (WT) mice, AD mice, Lcn2-deficient mice, and Lcn2-deficient AD mice. When they were 12 months of age, the mice were studied in different
behavioral and cognitive tests. Subsequently, mice were sacrificed, after which their brain tissue was studied for AD-like pathology.

**Behavior and cognition.** As expected [53–57], J20 AD mice showed AD-like behavioral and cognitive changes as compared to WT mice, including hyperactive locomotor behavior, and impaired hippocampus-dependent memory functioning. No differences were found between AD and Lcn2-deficient AD mice, indicating that Lcn2 does not significantly affect AD-like behavioral and cognitive changes in this mouse model of AD at 12 months of age. Of note, although AD and Lcn2-deficient AD mice were on average equally hyperactive, it appears that AD mice may be significantly more hyperactive at some specific times of day. These potential differences should be explored further, along with other aspects of circadian rhythmicity such as potential AD-like fragmentation of activity.

**Aβ plaque load and neuroinflammation.** As expected [53,56,58], AD mice presented clear Aβ plaque pathology and increased microglial and astrocyte activation (indicating neuroinflammation), as compared to WT mice. No differences in these AD-like pathological characteristics were found between AD and Lcn2-deficient AD mice. It should be noted that Aβ plaque pathology was only measured by analyzing the total surface covered by plaques, without e.g. counting plaques and distinguishing between small and big plaques. As such, it is possible that potential Lcn2-mediated differences in plaque numbers and sizes (and e.g. levels of Aβ1-42 and Aβ1-40) have been overlooked. The lack of differences in glial activation between AD and Lcn2-deficient AD mice is not in line with many publications in which other brain conditions were studied, which indicated that Lcn2 significantly promotes glial activation [6,7]. The reason for these contradictory findings is not clear, but may depend on e.g. the disease that is studied, the used animal model, the chronicity of Lcn2 exposure and age of the animals, as will be discussed further later in this discussion. It should be pointed out that only microglia and astrocyte activity were addressed in our mouse study. In future experiments it would be of interest to also study the possible involvement of infiltrated peripheral immune cells, such as neutrophils.

**Iron metabolism.** Iron accumulation was found in Aβ plaques in the hippocampus of AD mice, which corresponds to the human condition [59]. Moreover, iron appeared to accumulate in neurons, in the neuronal layers of the hippocampus. However, the exact brain cell types (and brain regions) in which iron accumulates should be established in future work. Interestingly, the extent of iron accumulation was less severe in Lcn2-deficient AD mice, indicating that Lcn2 may contribute to pathological iron accumulation in AD. This finding may be important, since excessive levels of reactive (labile) iron may contribute significantly to AD pathology, for instance via provoking oxidative stress and neuronal loss (e.g. due to ferroptosis) [3,59]. The mechanisms via which Lcn2 influences brain iron homeostasis, and the involved cell types, should be explored further. In the periphery it is known that Lcn2 under inflammatory conditions contributes to iron withdrawal from the circulation (leading to the so-called anemia of inflammation) [60,61], which likely protects against microbial infections by making iron less readily available for microbes [62]. This iron-retentive response is mediated by specific cell types (including liver cells and macrophages), which are able to
collect and accumulate high levels of iron [62]. It might be that a similar iron-retentive response occurs in the brain under neuroinflammatory conditions. Possibly, Lcn2 mediates the redistribution of brain iron under neuroinflammatory conditions, by promoting the uptake and retention of iron in specific brain cell types. Lcn2-mediated iron retention seems to take place in hippocampal neurons, as indicated by our iron staining results, and might also be expected to occur in microglia, considering the functions they share with macrophages. Indeed, neurons and especially microglia were described to accumulate high levels of non-transferrin bound iron under inflammatory conditions [63–67], which might in part be mediated by Lcn2. Astrocytes may be less prone to (Lcn2-mediated) accumulation of iron (as was also suggested by our findings in chapter 4) [64,66], yet more thorough investigation is required to establish this. In addition, it appears from our findings that Lcn2 may promote iron accumulation in Aβ plaques. Hypothetically, the antimicrobial effects that have previously been described for Aβ [43,44] might in part rely on the ability of Aβ to bind and collect iron, thereby reducing available iron that could otherwise be utilized by microbes. The Lcn2-mediated accumulation of iron in the diseased brain as described in this thesis has only been indicated once before, in a mouse model of intracerebral hemorrhage [30]. As such, our findings present a novel exciting effect of increased Lcn2 levels, which may be important in AD pathology and should be explored further. Nevertheless, it should be kept in mind that although Lcn2 aggravated AD-like pathological iron accumulation, this Lcn2-mediated iron accumulation was not severe enough to also induce significant changes in behavior, cognition, plaque load and glial activation, as compared to Lcn2-deficient AD mice. This may mean that in our AD model, accumulating iron levels did not yet exhaust safe iron storage facilities and compensatory (e.g. antioxidant) mechanisms. Possibly, in older age or in more aggressive AD mouse models, the dysregulation of iron metabolism will be more severe and will affect other AD-like pathological processes, including neuroinflammation, oxidative stress and cell death.

**Body weight.** One other interesting difference was found between AD mice and Lcn2-deficient AD mice, which was not yet shown in this thesis. Namely, the body weight of J20 AD mice was significantly lower than that of WT and Lcn2-deficient AD mice (also see Fig. 2). A significantly lower body weight was previously described for other AD mouse models, including APP23, APP/PS1 and 3xTg AD mice [68–70]. Interestingly, while body weight was decreased in these AD mice compared to WT mice, their food intake was increased (and metabolic rate was increased in 3xTg AD mice), indicating a hypermetabolic state [68–70]. In the current study we did not measure food intake or other measures of energy metabolism, but it may be hypothesized that also J20 AD mice present a hypermetabolic state. Together with the locomotor hyperactivity in J20 mice, this may explain the reduced body weight of J20 AD mice. The marked reduction in body weight, possible changes in energy metabolism and locomotor hyperactivity should be taken into account in the interpretation of results (and in future study designs), because they might significantly affect the behavior and physiology of J20 mice. Moreover, since our Lcn2-deficient AD mice do not show a decreased body weight compared to WT mice, it would be interesting to determine how the absence of
Fig. 2 J20 AD mice are significantly lighter than WT, Lcn2-deficient (Lcn2 knock-out; Lcn2 KO) and Lcn2-deficient J20 AD (J20xLcn2 KO) mice, at 12 months of age. J20 AD mice are on average ~5 grams lighter than WT mice (J20 AD: average of 34.6 g, WT: average of 39.7 g). Lcn2 KO mice (average of 41.4 g) and J20xLcn2 KO mice (average of 40.0 g) are similar in weight to WT mice. Tested with one-way ANOVA with Tukey’s multiple comparisons test. n= 13-17 mice per group (WT n=17, J20 n=13, Lcn2 KO n=16, J20xLcn2 KO n=16). * p < 0.05 and ** p < 0.01.

Lcn2 in Lcn2-deficient AD mice may prevent/rescue the otherwise lower body mass of AD mice. Interestingly, Lcn2 was previously shown to affect energy metabolism and to suppress appetite and food intake [32,71,72]. Taking these previous findings into account, Lcn2 is potentially involved in the apparent struggle of J20 AD mice to meet their great energy intake demands (for example by contributing to a potential higher metabolic rate in J20 mice, and suppression of sufficient appetite and food intake). Accordingly, absence of Lcn2 in Lcn2-deficient AD mice might slow down their energy metabolism, and/or increase food intake to such an extent that these mice manage to achieve a normal body weight. It should be mentioned however that several contradictory findings regarding the effects of Lcn2 on energy metabolism have been reported in previous studies. This for example includes contradictory effects of Lcn2 on body weight, food intake, glucose tolerance, insulin sensitivity, fat mass and thermogenesis (reviewed in chapter 6). These dissimilarities might depend on part on differences in diet, age, sex, genetic background and inflammatory state (and Lcn2 expression levels) of the studied mice, and potentially also on ambient temperature in the housing room of the mice. Altogether, the potential effects of Lcn2 on (AD-related changes in) energy metabolism would be interesting to investigate further in future research.

General considerations – Studying Lcn2, AD and other chronic age-related brain diseases
A few more general points of discussion should be mentioned, that may be taken into account in future studies of chronic age-related brain diseases including AD, and the effects of Lcn2 herein.
Human condition vs animal models

Considering the investigation of the neurobiology of chronic age-related brain diseases such as AD, important differences between human conditions and animal models thereof should be recognized. For example, the role of Lcn2 was often studied in acute animal models (e.g. by a single injection of a neurotoxic compound), while the respective human conditions are chronic in nature. In these acute models, usually only short-term effects of Lcn2 overexpression are studied, and as such the effects of chronically increased Lcn2 levels are not investigated. This is an important drawback of acute vs chronic disease models, since chronically increased Lcn2 levels might provoke different effects than short-term increased Lcn2 levels (as was also shown for other inflammatory factors, such as tumor necrosis factor alpha (TNF-α) [73–75]). Moreover, the expression levels of Lcn2 can differ tremendously between acute and chronic disease models. For example, Lcn2 expression in the brain was much higher upon intracerebroventricular injection of Aβ (an acute AD mouse model) as compared to APP/PS1 mice (a chronic AD mouse model) [18]. This may be explained by the fact that in the chronic mouse model of AD, mice have been exposed to increased Aβ throughout life. This long-term exposure to Aβ might have desensitized or exhausted brain immune cells as compared to acute exposure of Aβ, resulting in less strong but chronic Lcn2 overexpression [18]. This may explain in part why no significant effects of Lcn2 on glial activation were found in our chronic AD mouse model (chronic mild Lcn2 overexpression), in contradiction to the often strong pro-inflammatory effects of Lcn2 found in acute models (short-term high Lcn2 overexpression) of brain diseases. Another difference between human age-related brain diseases and animal models thereof is that the factor of aging is often not included in animal studies: animal studies regarding the role of Lcn2 in (age-related) brain diseases were often performed in young mice. Importantly, the entire state of the body and brain (including the brain immune system) is likely to be different in acute vs chronic and young vs old disease models, which may significantly influence how the body and brain respond to increased Lcn2 levels. As such, in future work it would be of great interest to study more (chronic) animal models of age-related brain diseases, at young as well as older ages (Fig. 3). Of note, it would herein be important to determine whether brain Lcn2 concentrations are comparable between human disease and chronic animal models of disease. Namely, it is imaginable that Lcn2 production may be higher in the human AD brain as compared to the chronic J20 AD mouse model, since many AD mouse models may display less severe neurodegeneration than that occurring in human AD [76]. In that case, besides Lcn2-deficient AD mice, Lcn2-overexpressing AD mice could be included in future investigations. In addition, it may be interesting to include certain risk factor conditions (e.g. unhealthy diet and physical inactivity) in these models, to more closely resemble the complex nature of human sporadic brain diseases (even though most chronic models mimic familial disease). Finally, it is important to note that although mouse and human Lcn2 are homologous, they are not identical [50,77]. While several characteristic functions may be comparable (e.g. antibacterial actions [78,79]), it should be taken into account that certain functions and effects of Lcn2 in mice may not be translatable to humans, and vice versa.
Fig. 3 Effects of Lcn2 may be significantly different between acute and chronic models of brain diseases, such as AD. In acute disease models, Lcn2 levels may become much higher than in chronic disease models, but are likely to normalize much faster. Short-term highly increased Lcn2 levels (in acute disease models, e.g. Aβ injection) may exert different effects than chronic mildly increased Lcn2 levels (in chronic disease models, such as transgenic J20 or APP/PS1 AD mice). As indicated by the question mark, it is not clear yet if brain Lcn2 concentrations are comparable between human AD and chronic AD mouse models.

General considerations in AD research
AD research in human AD patients and animal models of AD is often focused on the brain. However, it is clear that AD is also associated with pathological changes in the periphery of AD patients. For example, Aβ production/aggregation, inflammation and microbiome changes have been found to occur in different peripheral tissues and blood cells in AD patients and AD mouse models [52,80–85]. These AD-related pathological changes in the periphery may play an important role in the development and progression of AD pathology in the brain. Therefore, in future studies it would be valuable to not only study the brain, but also to gain further insight into the pathological processes that arise in the periphery, and the importance of these peripheral disease processes in the pathophysiology of AD. With regard to Lcn2, it would be of interest to study the potential involvement of Lcn2 in these peripheral pathological processes. For example, it may be hypothesized that Lcn2 could contribute to AD-related changes in peripheral energy metabolism, iron metabolism and microbiome changes in e.g. the gut and blood.

Additionally, while progressive neurodegenerative diseases such as AD become worse with time, it should be noted that certain disease processes in AD may not become increasingly severe as AD progresses, but may peak already in earlier stages of AD. For example, studies in human AD patients and mouse models of AD suggest that astrocyte activation might reach a maximum severity in relatively early AD stages, after which astrocyte reactivity may reach a plateau or become less severe (while e.g. Aβ pathology continues to worsen) [56,86–90]. Since astrocytes appear to be a major source of Lcn2 in the brain, it may be possible that the production of Lcn2 peaks already during earlier AD stages. As such, the
effects of Lcn2 in AD pathology may be strongest in earlier AD stages, when astrocyte reactivity and Lcn2 levels are at their peaks. It would be interesting to explore this possibility. The differential kinetics of different AD-related pathological processes emphasizes the value of studying humans and mice at multiple ages, at different stages of AD.

Understanding the effects of Lcn2
Regarding the effects of Lcn2, it should be stressed that the (sometimes contradictory) effects of Lcn2 likely depend on a complex combination of factors. For example, iron-bound and iron-free Lcn2 (as well as e.g. MMP-9-bound and MMP-9-free Lcn2) may exert significantly different effects, depending on the function of Lcn2 that is studied and the context (e.g. involved tissues) at hand. To illustrate this, administration of iron:siderophore:Lcn2 complex was reported to disrupt thermogenesis and mitochondrial respiration, while iron- and siderophore-free Lcn2 did not affect these processes [91,92]. On the other hand, iron-free Lcn2 has been suggested to promote cell death in certain cell types, while iron-bound Lcn2 did not affect cell survival [93]. Additionally, Lcn2 was found to promote dendritic spine retraction in cultured hippocampal neurons, with a stronger effect mediated by iron-free Lcn2 as compared to iron-bound Lcn2 [26]. As such, the effects of Lcn2 may in part depend on the availability of its binding partners, which may rely in part on for example diet (e.g. siderophores – which are required by Lcn2 to bind iron – are derived partly from the diet). To better understand the effects of Lcn2, it may be important to analyze the relative abundance of different ligands of Lcn2 and ligand-bound states of Lcn2.

Of note, many studies regarding the effects of Lcn2 have relied on the use of Lcn2-deficient mice, in which the Lcn2 gene has been knocked out (Lcn2−/−). It is important to note that although Lcn2-deficient mice seem similar to WT mice, some significant differences have emerged. For example, Lcn2-deficient mice display differences in iron metabolism and synaptic plasticity, as compared to WT mice. Moreover, although some contradicting findings have been reported, Lcn2-deficient mice may present significant differences in energy metabolism, anxiety-like behavior and cognition (reviewed in chapter 6). These results indicate that basal Lcn2 levels under healthy conditions are necessary for normal functioning of different physiological processes. Although it seems that certain phenotypes of Lcn2-deficient mice may be overcome with age (chapter 3 and 6), it should in future studies always be taken into account that absence of Lcn2 may already under healthy conditions affect certain physiological functions.

Concluding notes
In conclusion, AD pathology is accompanied by increased Lcn2 levels in the brain. In the J20 mouse model of AD, Lcn2 contributes AD-like dysregulation of brain iron homeostasis and AD-like decreased body weight. However, Lcn2 does not affect AD-like behavioral changes, cognitive impairment, Aβ plaque load and glial activation. It is possible that Lcn2 indeed does not significantly affect overall AD pathology. Alternatively, it might be that stronger effects of Lcn2 may become apparent in other experimental settings. In this regard, it would be of
interest to study the effects of Lcn2 over different ages (including young as well as older mice) in different chronic AD mouse models (ideally comparing AD mice with Lcn2-deficient as well as Lcn2-overexpressing AD mice), possibly while including risk factor conditions (such as unhealthy diet and physical inactivity). These future studies will be necessary to clarify the effects and importance of Lcn2 in AD pathology. Moreover, more work is required to gain a better understanding of the factors that influence the functions of Lcn2. For example, the effects of Lcn2 may depend significantly on the cell types by which it is expressed, the cell types that respond to it, the receptors via which it acts, the ligand-bound state of Lcn2 (e.g. iron-bound or iron-free), the levels of Lcn2 expression, the chronicity of Lcn2 exposure and the context (e.g. aged, inflamed, infected and/or otherwise diseased tissue, in male or female mice). Future studies will elucidate whether Lcn2 could serve as a promising therapeutic target and diagnostic marker for AD.
References


Chapter 7


General discussion


Chapter 7


