Comparing and contrasting white matter disorders: a neuropathological approach to pathophysiology

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2015

document version
Publisher's PDF, also known as Version of record

Chapter 6

Summary and general discussion

Comparing and contrasting white matter disorders: a neuropathological approach to pathophysiology

The first aim of this thesis was to ascertain if and to which extent the neuropathological evaluation of post-mortem tissue of patients with genetic white matter disorders can *per se* help identifying the neural cell type primarily involved in a given condition and the consequences on other cell types, and if this approach allows to categorize the neuropathological findings in individual diseases. The second aim was to define if this cellular pathology perspective is useful in identifying common pathomechanisms underlying the dysfunction or degeneration of the white matter, and also in assessing the extent of white matter repair potential and of the obstacles to it. Given the opportunity of studying two white matter disorders in parallel on human tissue and animal model systems, a third collateral aim was to acquire food for thought on the impact of phylogeny and development on the characteristics / behavior of white matter cell types in physiologic and pathologic conditions. As this approach repeatedly conflicted with the traditional classification of genetic white matter disorders, the final aim was to propose a novel cell-based classification which recapitulates all the above information. In facing the possibility to treat white matter disorders with cell-based therapies, a cellular pathology perspective needs to come into play.

For the purpose of the above mentioned aims, a selection was made of genetic white matter disorders of different aetiology investigated at the Center for Childhood White Matter Disorders of the VU University medical centre since 2010. This selection includes VWM (chapter 2), MLC and ClC-2-related disease (chapter 3), a hypomyelination syndrome (chapter 4), and two vascular leukoencephalopathies (chapter 5). Some of the diseases described are novel conditions.

Applying a cellular pathology perspective to the neuropathological evaluation of genetic white matter disorders

In routine practice, the neuropathological evaluation of a patient with a white matter disorder is mostly descriptive. It consists in the annotation of determinate macroscopic and microscopic features, which allow the diagnosis of a primary white matter disorder or, upon the recognition of certain clues, the identification of a definite disease. Genetic white matter disorders typically feature lack of myelin and white matter atrophy. Axons are expected to be spared or display a loss commensurate to myelin loss, and astrocytes are predicted to be reactive. Abundance of macrophages engulfed with myelin debris suggests ongoing active loss of myelin, whereas the presence of ischemic and necrotic lesions strongly points in the direction of a vascular disease. While useful in recognizing disorders the features of which are already defined, this diagnostic work-up may be of limited help in identifying clues to the pathophysiology especially of etiologically still undefined conditions. We hypothesized that, integrated into the routinely examination, a cellular pathology perspective is more effective in categorizing the main neuropathological features in individual cases and provides additional insights into the pathomechanisms driving white matter dysfunction and degeneration.

Traditionally, white matter disorders were considered diseases of myelin and of myelin-forming cells, the oligodendrocytes. This view has been challenged by the recent identification of leukoencephalopathies due to mutations in gene-products specifically expressed in cell types other than oligodendrocytes, and by the recognition of the massive influence that other cells types have on oligodendrocytes, myelination and myelin maintenance (chapter 1). A cellular pathology approach thus implies the recognition of the neural cell type that is mainly affected in the single case. Analysis of post-mortem tissue obtained at end stage disease may complicate this operation, dictating special attention in dissecting all secondary degenerative changes. In addition, the prior knowledge that a disease is due to mutations in gene products expressed by non-oligodendrocyte lineage cells may infer an unjustified bias in the search for the pathomechanisms of white matter degeneration. AxD exemplifies this issue. AxD is caused by dominant mutations in the astrocyte-specific gene product glial fibrillary acidic protein (GFAP) (Brenner et al., 2001). In children, *GFAP* mutations cause a leukoencephalopathy characterized by lack of myelin and, in severe cases, gelatinous dissolution of the white matter (Mignot et al., 2004). This coincides with accumulation of GFAP and heat shock proteins in astrocytes, forming inclusions

referred to as Rosenthal fibres (Iwaki et al., 1989; Mignot et al., 2004). There are different mouse models of Alexander disease, engineered to overexpress the murine or human, wild-type or mutant GFAP. These mice develop Rosenthal fibres in astrocytes at appropriate brain locations and become epileptic, as patients may be. Their white matter is, however, normal (Messing et al., 1998; Hagemann et al., 2006). Notably, these mouse models have been mostly employed to investigate the biologic impact of GFAP overexpression on astrocytic functions, including metabolic homeostasis of action potential-related glutamate release (Tian et al., 2010, Messing et al., 2010 & 2012). Little if any attention has been given to the effects of GFAP overexpression on developmental myelination and myelin maintenance. As to now, whether astrocytic Rosenthal fibres indeed contribute to the white matter degeneration has not been satisfactorily investigated in the mouse or in human tissue.

Vanishing white matter

We first applied a cellular pathology perspective to VWM. VWM is a genetic disorder affecting patients of all ages and due to mutations in any of the genes encoding the five subunits of the eukaryotic translation initiation factor eIF2B, a crucial regulator of protein synthesis under normal and stress conditions (Leegwater et al., 2001; van der Knaap et al., 2002). Although eIF2B is ubiquitously expressed, VWM is mainly or exclusively a leukoencephalopathy (chapter 1; van der Knaap et al., 2006). As illustrated in chapter 2.1, the neuropathology of VWM is characterized by lack of myelin and white matter rarefaction or cavitation. Typical of VWM are the presence of only few astrocytes that have an abnormal morphology with blunt cell processes, and of markedly increased numbers of oligodendrocytes. The first studies focused on oligodendrocytes in VWM and on the mechanisms underlying oligodendrocyte proliferation and demise (Rodriguez et al., 1999; Wong et al., 2000; van Haren et al., 2004). By contrast, we argued that oligodendrocytosis is rather a generic response to loss and lack of myelin, whereas the meager reactive gliosis and, in particular, the abnormal astrocytic morphology are more peculiar and characteristic of this disease. On these bases, we first hypothesized that astrocytes are the cell type bearing the largest functional brunt and, as such, driving the other aspects of VWM neuropathology.

Analysis of post-mortem human tissue showed that, in contrast with previous *in vitro* data (Dietrich et al., 2005), the meager reactive gliosis observed in VWM is

not due to deficient astrocyte generation. As exposed in chapter 2.2, white matter resident astrocytes actively proliferate and some may even derive from astrocytic differentiation of glial restricted progenitors, as suggested by the co-expression with GFAP of putative oligodendrocyte-lineage markers as the transcription factor olig2 and the galactolipid GalC (chapter 2.2; Cassiani-Ingoni et al., 2006; Magnus et al., 2007). These cells however remain immature, and many retain the immunoprofile of astrocyte precursor cells (chapters 2.2 and 2.3). Another peculiar aspect of the astrocytic pathology in VWM is the abnormal alternative splicing of GFAP. GFAP has different isoforms. In the brain, GFAPα is the most abundantly expressed GFAP isoform (Kamphuis et al., 2012). The alternative isoform GFAPδ differs from GFAPα in its unique carboxy-terminus (Middeldorp & Hol, 2011). GFAPα has the best intrinsic capacity to form filaments, whereas increased expression of other isoforms, including GFAPδ, yields condensed cytoskeletal networks (Kamphuis et al., 2012). VWM astrocytes show specific overexpression of GFAPδ, but not GFAPα, resulting in increased GFAPδ/α ratio. This finding was later confirmed by others (Huyge et al., 2012). Until now, VWM is the only human disease showing absolute GFAPδ overexpression with unchanged total GFAP and GFAPα.

Alternative splicing is the process by which alternative exons are recognized by the splicing machinery, incorporated in the mRNA and translated in a differential isoform. Alternative splicing is regulated by the strength of splice sites. Alternative exons as exon7+ of GFAP, expressed by the GFAPδ isoform, contain weaker splice sites with lower binding potential for the splicing machinery and are less often included in the mature transcript. Trans-acting proteins that bind to the pre-mRNA also regulate alternative splicing. They include the SR family of proteins, splicing activators that promote the insertion of alternative exons, and splicing repressors as the heterogeneous nuclear ribonuclear proteins (hnRNPs) that inhibit the inclusion of alternative exons by inhibiting SR protein binding (Wahl et al., 2009). In the human developing and adult brain, GFAPδ is highly expressed in neural stem cells in the subventricular and subgranular zone, and its expression declines in mature non-neurogenic astrocytes concomitant to increased expression of GFAPα (Roelofs et al., 2005; Middeldorp et al., 2010). Thus astrocytic differentiation and maturation coincide with a decrease in GFAPδ/α ratio, and this developmental switch in GFAP isoform ratio is supported by Notch activity (Kanski et al., 2014a). This suggests that GFAP alternative splicing plays a role in the maturation of astrocytes. Impaired developmental switch from a high to a low

GFAPδ/α ratio may cause defects in astrocyte differentiation. Since a high GFAPδ/α ratio resembles an immature isoform pattern (Kanski et al., 2014a), it can be speculated that deregulation of GFAP splicing contributes to restraining VWM astrocytes from maturation.

In mature astrocytes, GFAP alternative splicing is regulated by transcriptional and epigenetic factors. Inhibition of histone deacetylases (HDACs) decreases transcription of GFAP and enhances alternative isoform expression, resulting in increased GFAPδ/α ratio. Expression of GFAPδ is dependent on the presence and binding of splicing factors of the SR protein family (Kanski et al., 2014b). These findings may also provide important clues as to the pathophysiology of VWM astrocytes. In brain tissue of VWM patients, increased GFAPδ expression correlates with reduced levels of hnRNP mRNA (Huyghe et al., 2012). This suggests that deregulation of GFAP splicing in VWM may occur at an epigenetic level, with low hnRNP expression leading to increased SR protein binding to exon7+ and subsequent induction of GFAPδ expression. Importantly, HDACs inhibition increases GFAPδ/α ratio also in the presence of reduced GFAP transcription, and leads to aggregation of the GFAP intermediate filament network (Kanski et al., 2014b). This is in line with our observations on VWM human tissue (chapters 2.1 and 2.2). VWM white matter astrocytes fail to upregulate GFAP expression upon injury and have an abnormal morphology reminiscent of cells manipulated *in vitro* to incorporate excessive GFAPδ in an existing GFAP network (Nielsen et al., 2002; Roelofs et al., 2005; Perng et al., 2008). Interestingly, hippocampal astrocytes forced to overexpress wild-type and mutant GFAP in a cross between two Alexander disease mouse models show similar morphologic changes (Sosunov et al., 2013).

Consistent with an incorporation of excessive GFAPδ in the GFAP network, VWM astrocytes also accumulate αB-crystallin. αB-crystallin is a small heat shock protein belonging to the chaperone machinery that binds and stabilizes unstable conformers of other proteins (MacRae, 2000). It has been of particular interest for astrocytes, since it was found to be a major component of Rosenthal fibers in AxD (Iwaki et al., 1989). αB-crystallin promotes disassembly of preformed GFAP filaments and disaggregates Rosenthal fibers *in vitro* (Koyama & Goldman, 1999), suggesting that its overexpression in AxD is part of a stress response aiming at protecting the cell from the accumulation of Rosenthal fibers. Failure of this protective response could be due to depletion of αB-crystallin secondary to its sequestration into GFAP aggregates. This was confirmed in mouse model systems

of AxD. Mice forming Rosenthal fibers from overexpression of wild-type human GFAP have greater mortality when crossed into a αB-crystallin-null background (Hagemann et al., 2009). More importantly, constitutive overexpression of αB-crystallin in astrocytes results in a complete rescue from otherwise 100% mortality in a cross between two AxD mouse models (Hagemann et al., 2009). These observations open to the possibility that forcing αB-crystallin overexpression in VWM astrocytes may promote disaggregation of excessive GFAPδ and improve astrocytic function and clinical phenotype. Such a result would establish GFAPδ accumulation as a central pathomechanism in VWM.

The hypothesis that astrocytes play a central role in the pathophysiology of VWM was confirmed and further explored in the VWM mouse models characterized in chapter 2.4. Two transgenics were generated by insertion of homozygous VWM-causing mutations in two different *EIF2B* genes. The choice of the mutation was driven by the correspondent human phenotype. Both mice have a progressive neurological phenotype dominated by cerebellar ataxia similar to VWM patients. Breeding the two VWM mouse strains into heterozygous/homozygous or double-homozygous animals allowed reproducing the whole phenotypic spectrum of the human disease VWM within a mouse life, from the very severe form with early-onset and early mortality to the late-onset form with milder phenotype and longer survival. This strategy also enabled investigating which aspects of the astrocytic pathology correlate with disease severity and confirmed the negative impact that VWM astrocytes have on other neural cell types.

The typical features of human VWM astrocytes are recapitulated in all VWM mouse models. Astrocytic immaturity, manifested by the appearance and numbers of cells in the corpus callosum overexpressing the early intermediate filament nestin, is the first histopathological sign of the disease, and correlates with disease severity and progression. Nestin-expressing immature astrocytes overexpress GFAPδ, but not total GFAP or GFAPα. Contrary to humans, GFAP isoform stoichiometry in mice remains stable throughout astrocytic differentiation and during development, ageing and disease (Mamber et al., 2012; Kamphuis et al., 2012). GFAPδ overexpression is therefore an exquisitely pathological finding in mice, and indicates that deregulation of GFAP splicing is pathogenetically related to VWM. Notably, only immature white matter astrocytes overexpressing GFAPδ have blunt cell processes, confirming our previous observation in human VWM tissue that these different aspects of astrocytic pathology are related (chapter 2.3).

The central role of astrocytes in VWM pathophysiology is further supported by the finding that other types of astrocytes are affected in addition to white matter astrocytes (chapter 2.4). VWM mice have abnormal Bergmann glia in the cerebellar cortex, absent at birth but already evident before the onset of symptoms. These abnormal glial cells are displaced to the molecular layer, withdraw their endfeet at the pial surface and show a profound disorganization of cell processes. Notably, the abnormal Bergmann glia overexpress GFAPδ like white matter astrocytes. Remarkably, re-examination of the cerebellar histopathology of our brain collection revealed ectopic Bergmann glia also in VWM patients (chapter 2.4), and not in other genetic white matter disorders. Human abnormal Bergmann glia also overexpress GFAPδ, again supporting the hypothesis that aberrant GFAP splicing is a central pathomechanism in VWM across species. VWM mice also develop signs of laminar retinal disorganization that parallel the severity of the neurologic phenotype. The histopathological features are those of a retinal dysplasia with ectopia of inner nuclear and granule cells and disruption of the photoreceptor layer. All mutants display loss of glutamine synthetase immunoreactivity in Müller glia, which parallels the degree of clinical severity. In the more severely affected double-homozygous VWM mouse, Müller glia cells have an abnormal morphology with thick cell processes spanning through the disorganized retinal layers. Re-evaluation of the electroretinographic (ERG) data of VWM patients revealed preservation of the a-wave with loss of the b-wave of the trace. The ERG a-wave reflects the activity of photoreceptors and is largely independent of Müller cell activity. The b-wave is mostly generated by outer nuclear(ON)-bipolar cells. ON-bipolar cells are driven by photoreceptors via a glutamatergic pathway and glutamate released by photoreceptors is mostly taken up by Müller glia. In case of malfunctioning of the glutamate re-uptake transporters, including when Müller cells are not functional (Eckstein et al., 1997), glutamate levels increase in the outer retina and saturate the glutamate receptors of bipolar cells. As a result, bipolar neurons cease responding and the ERG b-wave is decreased (Eckstein et al., 1997). These combined mouse and human data indicate that retinopathy is also part of the human VWM phenotype and that it can be ascribed to an involvement of the retinal Müller glia.

Availability of the VWM mouse models characterized in chapter 2.4 helped confirm the hypothesis formulated in chapters 2.2 and 2.3 that other aspects of the VWM neuropathology are secondary to the major involvement of astrocytes.

In brains of VWM patients, lack of myelin coexists with increased numbers of oligodendrocytes (chapter 2.1; van der Knaap et al., 2006). As reported in chapter

2.2, this apparent contradiction prompted us to investigate if oligodendrocytes are impeded from maturing into myelin-forming cells. We found that the VWM white matter harbors increased numbers of cells with small cell bodies and multiple branched fine radial processes that co-express the oligodendrocyte-specific lineage marker Olig2 with PDGFRα or NG2 and that continue proliferate at a higher rate than in controls. These cells are proliferating OPCs. Reactive expansion of the OPC pool is not specific to VWM. Adult OPCs proliferate in response to acute white matter injury and can actively generate new mature oligodendrocytes in early MS lesions (Raine & Wu, 1993). OPCs may also proliferate upon chronic white matter injury as in chronic-active lesions in MS (Franklin & ffrench-Constant, 2008), ischemic white matter injury (chapters 2.3 and 5.1; Back et al., 2011; Buser et al., 2012) and different genetic white matter disorders, including AGS (chapter 5.2). In many chronic conditions, however, as in VWM remyelination does not occur and cells die by apoptosis (van Haren et al., 2004; Franklin & ffrench-Constant, 2008).

The hypothesis that the specific astrocytic pathology in VWM drives the maturation defect of OPCs was investigated in chapter 2.3. We argued that the immature VWM astrocytes may not be functionally competent to support OPC maturation and differentiation or may even hamper them. In an attempt to better characterize the degree of astrocytic immaturity, we found that many VWM astrocytes overexpress the astrocyte-restricted progenitor marker CD44 (Liu et al., 2004). CD44 is the membrane receptor of the glycosaminoglycan hyaluronan, a major component of the CNS ECM predominantly produced by astrocytes (Sherman & Back, 2008). Hyaluronan is known to inhibit OPC maturation in MS and ischemic white matter injury (Back et al., 2005; Back et al., 2011; Buser et al., 2012). Mice overexpressing CD44 under the control of a myelin-specific promoter accumulate hyaluronan and show myelin deficits (Tuhoy et al., 2004; Back et al., 2005). Quantification of hyaluronan in brain lysates from VWM patients revealed a massive hyaluronan overabundance with over 700-fold increase of the high-molecular weight species. Importantly, hyaluronan amounts, OPC numbers and degree of lack of myelin co-varied in differently affected white matter areas. These findings provided a first indirect, but strong suggestion that VWM astrocytes hamper OPC maturation and remyelination.

In chapter 2.4 we investigated the validity of this hypothesis in the VWM mouse models. The VWM mice are myelin deficient. At P21, an age at which developmental myelination is almost complete, VWM mouse myelin contains lower amounts of the major myelin protein MBP with a stoichiometric profile of MBP

isoforms typical of earlier developmental stages, indicating deficient myelin deposition. In this, VWM mice shed light on the substrate underlying the imaging findings of deficient myelination in young presymptomatic VWM patients (van der Lei et al., 2012). A similar deregulation of expression may also involve the other major myelin protein PLP (Huyghe et al., 2012). In older VWM mice, the amounts of MBP and other mature myelin proteins are more drastically reduced and the myelin is vacuolated. In general, the degree of myelin pathology correlates with disease severity and progression. In the more severely affected heterozygous/homozygous and double-homozygous mutants with greater lack of myelin the OPC numbers are increased. Overall, the VWM mice reproduce the myelin pathology of VWM patients. The hypothesis that myelination and OPC maturation may be negatively influenced by VWM astrocytes was tested *in vitro*. Co-culture systems of mutant and wild-type astrocytes and OPCs in variable combinations demonstrated that VWM astrocytes inhibit OPC maturation. Importantly, mutant OPCs mature normally when cultured with wild-type astrocytes. Co-cultures of wild-type cells grown in medium conditioned by mutant astrocytes reproduced the OPC maturation defect, whereas the maturation block caused by mutant astrocytes was lifted by medium conditioned by wild-type astrocytes. These data confirmed the hypothesis formulated for the human VWM, i.e. that the OPC maturation defect is not intrinsic to oligodendrocytes, but rather induced by astrocytes. Additionally, the conditioned medium experiments showed that astrocytes inhibit OPC maturation via secreted factors. In mice, however, hyaluronan seems to play a major role only at end-stage disease. Hyaluronan amounts were increased in brain lysates and conditioned medium of only some of the VWM mutants, and to a variable extent. Notably, even when increased, hyaluronan amounts in mice never reached the levels documented in patients (maximum 2-fold increase in mice, over 700-fold increase in patients). This observation, together with the co-variance of hyaluronan amounts and OPC numbers in differently affected white matter areas of VWM patients, suggest that hyaluronan is only one of different astrocyte-related factors impacting on OPC maturation in VWM. As illustrated in chapter 1, astrocytes impact on oligodendrocyte lineage cells in many ways and different astrocytic secreted factors have been identified that hamper OPC maturation, which are probably at play also in VWM. In addition, the pathologic microenvironment of VWM white matter could contain other not directly astrocyte-related clues that contribute to influence OPC behavior. OPC proliferation, for example, also occurs chronically in white matter of mice with genetic myelination defects as *shiverer* (Bu et al., 2004) and *jimpy* (Wu et al., 2000), despite elevated cell density. This has been shown to

be closely associated with lack of intact myelin (di Bello et al., 1999). A similar mechanism may also operate in VWM, in which both lack of myelin and myelin vacuolation are observed in patients (Rodriguez et al., 1999) as well as in mice (chapter 2.4).

Finally, we asked if VWM astrocytes also have a negative impact on axons. In VWM patients, neuropathology at end-stage disease shows axonal loss and reduced axonal caliber in the affected white matter (van der Knaap et al., 1998; van der Knaap et al., 2006; chapter 2.1). In the corpus callosum of VWM mice, axons are thinner and the percentage of thin axons is higher than in controls. Notably, the axonal cytoskeleton appears ultrastructurally normal. Myelin disease may secondarily affect axons. Axons and ensheating oligodendrocytes interact bidirectionally and, as exposed in chapter 1, oligodendrocytes play a role in modulating axonal caliber and supporting long-term integrity of axons (Nave, 2010; Funfshilling et al., 2012). In line with this, myelin deficient *shiverer* (Brady et al., 1999) and *jimpy* mice (Robain, 1977; Rosenfeld & Freidrich, 1983) also have thin axons. Clearly, further research is needed in order to address if and which role VWM astrocytes play in determining axonal pathology.

Overall, the available data show how a cellular pathology perspective integrated in the neuropathological examination of VWM proved useful in identifying (1) astrocytes as the cell type functionally primarily involved in the disease, (2) the myelin and oligodendrocyte pathology as a secondary phenomenon to the astrocytic dysfunction, and (3) specific aspects of the astrocytic pathology conceivably representing the pathomechanisms driving the white matter degeneration.

MLC and ClC-2-related leukoencephalopathy

Applying prospectively a cellular pathology perspective to the neuropathological examination of disorders of ion-water homeostasis in a blinded manner was not possible. Our tissue collection comprises only the brain biopsy specimen of one *MLC1*-mutated MLC patient and some tissue sections of another MLC patient with a dominant *GLIALCAM* mutation. However, analysis of these specimens at the light of the MLC, GlialCAM and CLC-2 expression data obtained on the *Mlc1*-null mouse and normal human tissue provides interesting clues as to the histopathological changes that could help addressing the diagnosis towards a defect of ion-water homeostasis.

The diseases due to defects of ion-water homeostasis analyzed in this thesis are MLC and ClC-2-related leukoencephalopathy. Both conditions are characterized clinically by a relatively mild clinical course and radiologically by white matter signal changes on MRI and diffusion parameters indicative of increased white matter water content (van der Knaap et al., 1995 and 2012; Singhal et al., 1996; van der Voorn et al., 2006; chapter 3.2). Published neuropathological data pertaining brain biopsies of MLC patients show countless fluid-filled vacuoles within the outer lamellae of myelin sheaths and, to a lesser degree, in perivascular astrocytic endfeet. These changes are accompanied by gliosis without overt myelin or axonal loss (Harbord et al., 1990; van der Knaap et al., 1996; Pascual-Castroviejo et al., 2005; Miles et al., 2009; Duarri et al., 2008). The neuropathology of ClC-2-related leukoencephalopathy is still undefined because *post-mortem* human tissue is to date not available.

In chapter 3.1 we characterized an MLC mouse model. This model was generated by replacing two exons of the *Mlc1* mouse gene with the coding region of the enhanced green fluorescent protein. Such a construct allowed confirming what was previously assumed based on human MLC1 expression data (Boor et al., 2007), *i.e.* that MLC1 is exclusively expressed by astroglial lineage cells, including astrocytes involved with fluid barriers, Bergmann glia and ependymal cells. This suggests that the neuropathological clues to MLC and perhaps to ion-water homeostasis defects in general may be found at these sites. Indeed, neuropathology of the *Mlc1*-null mouse showed peculiar astrocytic changes. Around blood vessels and, to a lesser degree, underneath the pial surface, astrocytes appeared hypertrophic. Of note, especially the cell processes abutting on blood vessels and brain surface were enlarged, a change that was clearly detectable both by electron and optic microscopy. The unchanged expression of GFAP, vimentin and nestin in these cells argues against hypertrophy in the context of reactive gliosis, and rather suggests cellular swelling. Meticulous retrospective evaluation of brain tissue of our two MLC patients revealed that swelling of perivascular and subpial astrocytic cell processes and endfeet is also evident in humans (fig. 1). Notably, one of these patients was a child with a dominant *GLIALCAM* mutation that is associated with improvement and often resolution of the clinico-radiological phenotype during the first few years of life (van der Knaap et al., 2010; Lopez-Hernandez et al., 2011). GlialCAM functions to ensure the proper localization of MLC in astrocytic membranes (Lopez-Hernandez et al., 2011). The observation of similar astrocytic changes in both MLC patients

suggests that minimal neuropathological changes of astrocytes persist in MLC due to dominant *GLIALCAM* mutations even with major improvement of the phenotype.

As in humans, abnormal astrocytic morphology in *Mlc1*-null mice was accompanied by intramyelinic edema (chapter 3.1). Neuropathological analysis of the mutant brains at different time points revealed that astrocytic abnormalities precede the appearance of myelin vacuolization. This finding suggests a pathologic sequence of astrocyte dysfunction and swelling leading to water retention and then spongiform myelin changes at later stages. Intriguingly, such sequence of events is in complete agreement with the role of astrocytes in potassium siphoning (van der Knaap et al., 2012; chapters 1, 3.1 and 3.2) and with the function of MLC1 in volume-regulated anion channel currents and regulatory volume decrease after cell swelling (Ridder et al., 2011; van der Knaap et al., 2012; chapters 1 and 3.1). On these bases, it can be suggested that the combination of astrocytic swelling at specific locations and intramyelinic edema is indicative of a defect in ion-water homeostasis.

Other genetic leukoencephalopathies are characterized by intramyelinic edema (van der Knaap et al., 2005; chapter 1). Although intramyelinic edema can be readily identified *in vivo* with diffusion MR imaging (van der Knaap et al., 2005; van der Voorn et al., 2006; chapter 3.2), the exact histopathological substrate of many conditions is not known due to lack of human tissue. In principle, in view of the mutant gene product, failure of potassium siphoning and loss of ion-water homeostasis can be envisioned for many of these disorders, including ClC-2-related leukoencephalopathy (chapter 3.2; di Bella et al., 2014), gap junction defects (chapter 3.2; Abrams et al., 2014) and possibly Canavan disease (Baslow, 2003; Baslow & Guilfoyle, 2013; Clarner et al., 2014). Whether this also applies to other genetic disorders with 'toxic' intramyelinic edema as phenylketonuria, non-ketotic hyperglycinemia, maple syrup urine disease, glutaric aciduria type I and urea cycle disorders is not known. It would be interesting to determine whether astrocytes in these disorders display the same suggestive morphological changes as in MLC. This observation has not been reported in the neuropathological description of these conditions (Martin & Schlote, 1972; Agamanolis et al., 1982 & 1993; Crome 1961; Kimura et al., 1994; Soffer et al., 1992; Ebels, 1972; Kornfeld et al., 1985), but it may have been overlooked as it happened to us for MLC (chapter 3.1). Intriguingly, however, in their original description of maple syrup urine disease, Menkes, Hurst and Craig described "swelling of the astrocytes in the corona radiata of the cerebral hemispheres" (Menkes et al., 1954).

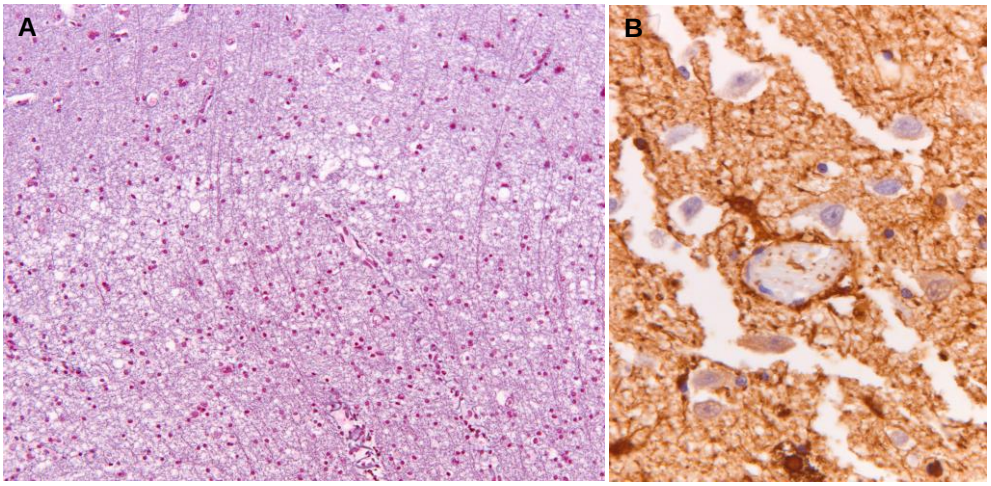

Figure 1. In the temporal pole of an MLC patient with a dominant *GLIALCAM* mutation a subtle vacuolation is focally present at the cortico-subcortical junction (A, Bodian), where perivascular astrocytes show thickened cell processes abutting blood vessels (B, GFAP).

The phenotype of the *Mlc1*-null mouse is characterized by brain swelling and intramyelinic vacuoles, thus reproducing the early presymptomatic stage of MLC patients. Comparing the temporal profile of MLC1 expression in control human and WT mouse tissue at the light of the clinical course in MLC patients and the evolution of the neuropathology of *Mlc1*-null mice shed light on the relation existing between MLC defect and myelin pathology. In both mice and men, MLC1 expression increases during rapid myelin deposition and white matter edema develops and is most pronounced when MLC1 expression is highest. This correlation between active myelination, defective MLC1 expression and severity of intramyelinic edema is relatively maintained across the two species, further supporting the role of MLC in ion-water homeostasis. Notably, in both species the myelin is vacuolated, but appears otherwise normal. Immunohistochemistry indicates normal expression of the major mature myelin proteins, a datum confirmed in *Mlc1*-null mice by Western blotting and mRNA analysis, and no myelin breakdown or increased OPC numbers are observed. Normal expression and localization of myelin proteins has also been observed in the *Clcn2*-knockout mouse (Blanz et al., 2007). Mice with astrocyte-targeted deletion of Cx43 or deletion of Cx30 have normal myelin, and double knockout of both Cx genes is necessary to impair OPC maturation and myelin deposition (Lutz et al., 2009). It could be speculated that leukoencephalopathies due to astrocytic ion-water

homeostasis defects, including MLC and ClC-2-related leukoencephalopathy, do not significantly affect myelin integrity, which could explain the observation that compared to the classical leukodystrophies and hypomyelinating diseases these disorders may be clinically less severe or even subclinical and more slowly to virtually non progressive (chapters 3.1 and 3.2; van der Knaap et al., 2012; Kocaman et al., 2013; di Bella et al., 2014; Abrams et al., 2014).

Comparing MLC patients with *Mlc1*-null mice and *CLCN2*-mutated patients with *Clcn2*-mutant mice provides an example of how the clinical expression of a genetic white matter disorder may differ across species. Mlc1-null mice have megalencephaly and intramyelinic edema, but no obvious motor impairments (chapter 3.1). *CLCN2* mutations cause a juvenile or adult-onset disease in humans (chapter 3.2), while *Clcn2*-mutant and *Clcn2*-knockout mice display intramyelinic edema, but no clinical neurological abnormalities (Bösl et al., 2001; Blanz et al., 2007; Edwards et al., 2010). While developmental leukodystrophies with defective myelin deposition are recapitulated by mutant mice as *jimpy* (Robain, 1977) and *shiverer* (Chernoff, 1981), mouse models for white matter disorders with onset after infancy often lack a clinical phenotype (Lu et al., 1997; Behrendt et al., 2014; Hagemann et al., 2006). The models of X-ALD (Forss-Petter et al., 1997; Kobayashi et al., 1997) and MLD (Hess et al., 1996), for example, show biochemical signs of disease, but lack the expected clinical phenotype. Major mouse-human species differences, including life span, amount of cerebral hemispheric white matter and differences in biochemistry are limiting factors (chapters 2.4, 3.1 and 3.2; Forss-Petter et al., 1997).

In case of defects in gene products involved in ion-water homeostasis, inter-species differences in expression of astrocytic proteins and heterogeneity of astrocytes may also account for the different regional involvement of the white matter. In MLC patients, white matter edema is most severe in the cerebral hemispheres followed by the cerebellum, whereas the corpus callosum and brainstem are relatively spared (van der Knaap et al., 1995). In *Mlc1*-null mice, the cerebellar white matter is first and most severely affected, but mice hardly have cerebral hemispheric white matter beyond the corpus callosum. Like humans, *Mlc1*-null mice show a greater involvement of the cerebellar white matter than the brainstem and corpus callosum. Within different brain regions, astrocytes express different levels and types of ion channels and thus have different electrophysiological properties and functions (Oberheim et al., 2012). MLC1 expression levels are higher in the mouse cerebellum than in other brain areas (Teijido et al., 2007). Strikingly, in the cerebellar Bergmann glia loss of MLC1

expression is accompanied by the most profound loss of GlialCAM and ClC-2 expression compared to other astrocytic populations and brain areas. It may, therefore, be that mouse Bergmann glia form a particularly vulnerable type of astrocyte prone to manifesting the consequences of loss of MLC1 function. Comparable human data are lacking. In the human normal white matter, ClC-2 shows diffuse membrane expression the panglial syncytium, with enrichment around blood vessels, in the glia limitans, ependymal lining, and astrocyte–astrocyte contacts, where it colocalizes with MLC1 and GlialCAM (chapter 3.2). In mice, by contrast, Clc2 expression in astrocytes is limited to the perivascular endfeet (Blanz et al., 2007). A similar polarized vs. diffuse expression in mouse vs. human astrocytes has also been described for the astrocytic water channel aquaporin4 (Skjolding et al., 2012) and could be envisioned for other astrocytic proteins involved in the control of water-ion homeostasis. When comparing phenotypic expressions and pathomechanisms across species (especially as distant as humans and mice), an integrated approach that takes into account evolution, development and pathology ("evo-devo-patho" approach) may be tremendously helpful in interpreting inter-species similarities and differences and obtain a better understanding of the pathophysiology of a disease.

Hypomyelination

The concept of hypomyelinating disorders is relatively recent. Since the 1980s, attention was progressively drawn on a category of genetic white matter disorders characterized by abnormalities in myelin development rather than destruction (van der Knaap & Valk, 1989; van der Knaap et al., 1999; Schiffmann & van der Knaap, 2009; Pouwels et al., 2014). Hypomyelinating disorders are more typically early-onset diseases recognized on MRI by mild T2 hyperintensity of the white matter, indicating lack of myelin, without the prominent T1 hypointensity typically seen with myelin loss (Steenweg et al., 2010). In clinical practice, MRI may further address the differential diagnosis by detecting involvement of selected white matter areas or other brain structures, and follow-up studies distinguish true hypomyelination from delayed myelination in younger children (Steenweg et al., 2010; van der Knaap & Wolf, 2010).

From a myelin-centric point of view, genetic hypomyelinating disorders have traditionally been ascribed to defects of myelin components, other intrinsic defects of oligodendrocytes or defects of the program regulating OPC specification, differentiation and maturation. Myelin deposition and maintenance, however, are

not oligodendrocyte cell-autonomous processes, and hypomyelinating disorders due to defects in the complex interaction between oligodendrocytes, astrocytes and axons are increasingly being recognized.

Defects of myelin-specific lipids and proteins were the first to be described and long considered to be the only cause of hypomyelination. PMD, due to changes in the *PLP1* gene, typifies this category. Neuropathology of PMD shows lack of myelin with relative preservation of axons, oligodendropenia and marked astrocytosis. Of interest, accompanying cerebellar cortical atrophy with granule or Purkinje cell degeneration is also observed (Seitelberger, 1970; Pamphlett & Silberstein, 1986). Oligodendropenia in PMD results from aggregation of mutant PLP1 at the endoplasmic reticulum or from accumulation of excessive PLP1 in the late endosome/lysosome compartment, both leading to oligodendrocyte loss by apoptosis (Torii et al., 2014). Thus, pathogenetically PMD is a disease of myelin *and* of the oligodendrocytes. A similar combination of lack of myelin, oligodendropenia, astrogliosis and cerebellar cortical degeneration is also observed in Cockayne syndrome, a hypomyelinating nucleotide excision repair-related disorder due to mutations in the *ERCC6* and *ERCC8* genes (Patton et al., 1989; Troelstra et al., 1992; Henning et al., 1995).

As outlined in chapter 1, early oligodendrocyte development is controlled by specific transcription factors that promote glial subtype specification of OPCs. Transcription factors as Olig2, Sox10, and Nkx2.2 are essential for this stage. Amongst these, only mutations in *SOX10* have been associated with a hypomyelinating disorder (Pingault et al., 1998). Other transcriptional proteins, chromatin remodeling and signalling pathways such as integrin and PI3 kinase promote later stages of oligodendrocyte differentiation and myelin remodelling. No hypomyelination-related mutations have been identified in these pathways, probably because they are essential to many different cell types. The regulation of the myelinogenetic program, however, is also dependent on clues that are extrinsic to the oligodendrocyte, and extra-oligodendrocytic regulators are likewise later involved in myelin maintenance (chapter 1). This suggests that, besides the primitive disorders of the myelin and oligodendrocytes, hypomyelination may also be secondary to defects in other cell types. Indeed, abnormal myelination is classically observed on MRI also in early-onset neurodegenerative diseases (Steenweg et al., 2010). Recent neuropathological descriptions suggest that a diffuse lack of myelin with paucity of oligodendrocyte lineage cells accompanies the neuronal and/or axonal changes in infantile-onset lysosomal neuronal storage disorders as GM1 gangliosidosis (van der Voorn et al., 2004). Earlier descriptions

of white matter changes in GM2 gangliosidosis (Haberland et al., 1973), infantile NCL (Haltia et al., 1973), Salla disease (Autio-Harmainen et al., 1988) and fucosidosis (Bugiani & Borrone, 1976) usually refer to white matter “demyelination”, “dysmyelination” or ”degeneration” often with gliosis. Loss of oligodendrocytes, “glial cell degeneration” and “absence of interfascicular oligoglia” is also mentioned. The concept of hypomyelination was already cautiously suggested with statements as “there are some reasons to argue that the oligoglia … are probably unable to perform myelination or to maintain myelin sheaths” (Bugiani & Borrone, 1976). It should be noted that these descriptions precede the delineation of hypomyelination as a separate white matter disease category. Specifically interrogated on the subject, the author of one such description replied that, applying the current knowledge and criteria, the white matter changes in fucosidosis would now be described as secondary hypomyelination (Bugiani O, personal communication). Additional new hypomyelination syndromes due to changes in gene products expressed also or solely in neurons and axons have been identified by means of MRI pattern recognition combined with whole exome sequencing. These include disorders related to *TUBB4A* (hypomyelination with atrophy of the basal ganglia and cerebellum [H-ABC] [Simons et al., 2013]; isolated hypomyelination [Pizzino et al., 2014]), *AGC1* (global cerebral hypomyelination [Wibom et al., 2009; Wolf & van der Knaap, 2009]), *HSPD1* (Magen et al., 2008), and *AIMP1* (Feinstein et al., 2010; Boespflug-Tanguy et al., 2011). These disorders still await a neuropathological description due to lack of post-mortem material.

More recently, a third category of hypomyelination disorders has been described due to defects in gene products involved in protein synthesis, e.g. the aminoacyl-tRNA synthetase (ARS)-related disorders and RNA polymerase III (Pol III)-related disorders.

ARSes are essential enzymes responsible for performing the first step of protein synthesis. Specifically, ARSes attach amino acids to their cognate tRNA molecules in the cytoplasm and mitochondria (Antonellis & Green, 2008). Despite their critical role in protein synthesis and ubiquitous expression, mutations in genes encoding cytoplasmic and mitochondrial ARSes may result in restricted neurological phenotypes, raising questions about the role of these enzymes (and RNA translation and protein synthesis in general) in neural cell function. Defects in two cytoplasmic ARSes have to date been associated with hypomyelination: *RARS*, encoding the arginyl-tRNA synthetase (hypomyelination with nystagmus and leg spasticity [Wolf et al., 2014a]) and *DARS*, encoding the aspartyl-tRNA synthetase (hypomyelination with brainstem and spinal cord involvement and leg spasticity

[HBSL] [Taft et al., 2013]). Interestingly, both *RARS* and *DARS* gene products are part of a multi-enzyme (multi-synthetase) complex consisting of nine different ARSes and three auxiliary proteins (Wolfe et al., 2005). Of note, one of such auxiliary proteins is the above-mentioned AIMP1, a neuronal protein the defects of which cause hypomyelination secondary to a neuronal disorder (Feinstein et al., 2010). The role of the multi-synthetase complex is not completely clear. Apart from tRNA and protein synthesis, the multi-synthetase complex may have other non-canonical functions, including maintenance of intracellular tRNA homeostasis, that appear to be crucial for myelination.

Failure to maintain tRNA homeostasis is likely also central in Pol III-related hypomyelinating disorders. Pol III is a DNA-directed polymerase involved in (the regulation of) transcription of noncoding RNAs and tRNAs (Dieci et al., 2007). Changes in POLR3A and POLR3B, forming the active core of Pol III, cause a hypomyelination syndrome variably associated with dental and endocrine abnormalities and myopia, named hypomyelination with hypodontia and hypogonadotropic hypogonadism (4H syndrome) (Wolf et al., 2005; Bernard et al., 2011; Saitsu et al., 2011; Tétreault et al., 2011; Daoud et al., 2011; Wolf et al., 2014b). In this thesis, we characterized the neuropathology of one 4H patient (chapter 4). The findings largely overlap with what recently reported for another patient (Vanderver et al., 2013), e.g. white matter atrophy with lack of myelin and marked oligodendropenia, axonal loss and relatively mild astrogliosis and microgliosis. Indulging in cell counting, Vanderver *et al.* quantified the loss of oligodendrocytes in the centrum semiovale and corpus callosum by a rough 70 to 90%. Notably, the two patients differ in some aspects. Vanderver *et al.* (2013) noted mineralization of cortical layers I and II, which was absent in our patient, whereas we observed loss of granular neurons and Purkinje cells with axonal and dendritic swellings in the cerebellar cortex that were not present in the other patient. The axonal damage in the cerebral white matter and cerebellar cortex points to an important neuroaxonal involvement associated with the hypomyelination in Pol III-related disorders. All brain disorders, however, including white matter disorders, involve neurons and axons at end-stage disease, a feature that provides little clues as to the primarily affected cell type. This is also observed in the prototypic oligodendrocyte disorder PMD, where neuroaxonal degeneration is a feature of late disease (Garbren et al., 2002). The cerebellar cortical involvement with changes and loss of granular and Purkinje cells observed in many (and not only) hypomyelinating disorders (Steenweg et al., 2010) is conceivably to be interpreted in this same perspective. Besides reflecting a hypothetical primary

degeneration of selectively vulnerable cell populations, cerebellar cortical atrophy could more likely be secondary to the lack of myelin. Notably, cerebellar cortical lamination is usually maintained even in early-onset disorders. This suggests that remote effects operating via trophic trans-synaptic interactions between the affected telencephalic white matter and the cerebellum likely account for this type of cerebellar cortical changes.

In hypomyelinating disorders, both primary and secondary to neuronal or axonal changes, the reported neuropathology thus shows lack of myelin with oligodendropenia and variable gliosis. Oligodendropenia could result from a defect in OPC proliferation or survival, either primary or secondary to axonal pathology that prevents OPCs from maturing into myelin-forming cells. This possibility has not been investigated. The growing knowledge on the critical role of proper axo-glial interactions during myelination (chapter 1) provides the frame in which to allocate the pathophysiology of secondary hypomyelination. Applying a cellular pathology perspective, and as banal as it may sound, oligodendropenia in the context of lack (and *not* loss) of myelin appears as the strongest neuropathological indicator of hypomyelination.

The role of astrocytes in influencing the fate of OPCs (chapters 1, 2 and 5) suggests that hypomyelination can be secondary also to astrocytic changes. The neuropathology of white matter disorders due to defects in astrocyte-specific gene products, as AxD, or to a predominant functional involvement of astrocytes, as VWM, supports this possibility. Interestingly, the pathomechanisms leading to the hypomyelination may somehow be influenced by the age of onset.

In AxD, a failure of normal myelination is most likely to be responsible for at least part of the white matter pathology (van der Knaap et al., 2001). In the infantile variant, the white matter is partially to completely cystic and shows extensive paucity of myelin with only scattered oligodendrocytes. Phagocytes are not increased and no sudanophilic breakdown products are usually seen. In non-cystic areas, axons are preserved (Wohlwill et al., 1959; Borrett & Becker, 1985, Towsend et al., 1985; Neal et al., 1992; Klein & Anzil, 1994). This combination of findings is more consistent with a failure of myelin formation than with myelin breakdown. In childhood onset cases, some degree of myelination occurs, as suggested by the relative preservation of U-fibers and presence of phagocytes containing neutral fats. Although oligodendrocyte loss is mentioned in some case reports, oligodendrocyte numbers, proliferation rate and maturation status have not been systematically assessed (Russo et al., 1976; Towfighi et al., 1983; Borrett &

Becker, 1985; Pridmore et al., 1993). In the very few cases of prenatal or very early-infantile onset VWM on record, the cerebral white matter shows near total absence of myelin and reduced numbers of oligodendrocytes (Boltshauser et al., 2002; Hata et al., 2014). We also found marked oligodendropenia in the hemispheric white matter of two VWM babies (unpublished observation). By contrast, in later-onset VWM variants, lack of myelin coexists with increased numbers of oligodendrocyte progenitors that are impeded from maturing into myelin-forming cells (chapters 2.1-2.3). Comparing AxD and VWM to the disorders illustrated above suggests that oligodendropenia underlies the hypomyelination of early-onset astrocytic diseases as it does in other hypomyelinating disorders, either primary or secondary to neuronal/axonal changes. Considering the oligodendrocytic density in function of the age, it can be speculated that dysfunctional astrocytes cause lack of myelin by interfering (either directly or indirectly via inducing axonal pathology) with OPC proliferation or survival during developmental myelination and by hampering OPC maturation later in life. In both cases, the expected end results are shortage of mature oligodendrocytes and lack of myelin.

Vascular leukoencephalopathies

In chapter 5, we describe the neuropathology of two genetic encephalopathies related to vascular injury. These diseases show very diverse etiology, age at onset and clinical course, but share a diffuse involvement of the white matter and thus provide the opportunity to consider the white matter pathology from a cellular perspective.

The neuropathology of the ischemic injury to the white matter has been object of detailed studies for a long time (Fern et al., 2014). Attention has been especially drawn to the white matter pathology associated with vascular diseases in adults and hypoxic-ischemic encephalopathy in infants. In both cases, the neuropathology of white matter lesions appears to reflect the severity of the ischemic insult. Severe insults trigger necrosis with pancellular degeneration and loss of glial cells and axons, whereas milder injury results in persistent lack of myelin and remyelination failure.

Chapter 5.1 describes a novel autosomal dominant vascular leukoencephalopathy with onset in adulthood. The disease is characterized clinically by cerebrovascular accidents and slow, late cognitive deterioration, associated with therapy-resistant hypertension, a peculiar facies, xerostomia and xerophthalmia, and muscle

cramps. The MRI pattern is distinctive in that the white matter signal changes become rapidly diffuse and the leukoencephalopathy precedes the onset of vascular accidents and is disproportionate to the degree of clinical severity. The previous report of another family with overlapping clinical and MRI features and sharing the same 4.5 Mb haplotype on chromosome 20q13.12 (Hervé et al., 2012) strongly supports that this is indeed a disease entity. To highlight the association of diffuse leukoencephalopathy and vascular lesions, we coined the acronym ADLAVA (autosomal dominant laeukoencephalopathy and vascular accidents). With whole exome sequencing, we identified two variants in two different genes. Both variants are predicted to be detrimental by *in silico* analysis; although the clinical phenotype and expression studies favor one over the other, for reasons that go beyond the scope of this discussion (but outlined in chapter 5.1) we could not definitely conclude which variant causes the disease.

Three affected family members were analyzed at autopsy and compared to other forms of small vessel disease (SVD), including sporadic hypertension-related SVD, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), and capillary cerebral amyloid angiopathy (CAA). Neuropathology showed hypertension-related vascular changes and possibly periventricular venous collagenosis with superimposed additional peculiar changes of the terminal arterioles and pre-capillary junctions that were absent in all other SVDs. The exact nature of these changes is still undefined; however they were also noted in the family reported by Hervé *et al*. (2012) and thus conceivably represent a feature of the disease. In the patients' white matter, ischemic lesions were no different from what is typically found in other SVDs, with loss of oligodendrocytes, myelin and axons often centred on abnormal blood vessels and lacunar infarcts (Jellinger, 2007). The pathogenesis of these lesions is heterogeneous and supposedly related to chronic ischemia with reduced blood flow due to arteriolar stenosis, hypertension-related structural changes of the vessel walls, altered blood flow autoregulation and loss of integrity of small vessel endothelium or the blood-brain barrier (Jellinger, 2007; Iadecola et al., 2010; Hachinski, 2007; Young et al., 2008). Notably, the better preserved white matter areas with no evidence of venous collagenosis or angiosclerosis still showed paucity of myelin with intense gliosis, conserved oligodendrocytic density and absence of phagocytes. The degree of myelin pallor was greater than in the other SVDs. This was also the case for the subcortical whiter matter (thus outside the areas commonly involved in leukoaraiosis) and for younger patient analyzed, who had few if any vascular lesions. We interpreted these findings as the

histopathologic substrate of the discrepancy between extensive white matter involvement and relatively mild vascular lesions observed on MRI, and argued that additional mechanisms besides ischemia and structural vascular changes contribute to the pathogenesis of the leukoencephalopathy. In particular, we questioned if astrocytes may play a pathogenetic role because of the intense gliosis observed in the non ischemic white matter.

We explored the expression of endothelin-1 (ET-1), a peptide with roles in vasoconstriction and oligodendrocyte development, secreted by astrocytes and endothelial cells and degraded by one of the mutated proteins (Itoh et al., 1995; Seyrantepe et al., 2008; Gadea et al., 2009). As previously described (Zhang et al., 1994), we found ET-1-immunopositive reactive astrocytes in and around vascular lesions in the white matter of patients as well as the other SVDs. In this context, it has been speculated that ET-1 released from astrocytes may reach intracerebral arterioles and induce long-lasting vasoconstriction, thus contributing to tissue ischemia (Zhang et al., 1994). By contrast, in non-ischemic white matter areas astrocytic ET-1 immunolabelling was strikingly increased in patients compared to all other SVDs. This indicates that, in our patients, astrocyte-derived ET-1 accumulation may not only be related to the vascular injury. Indeed, ET-1 immunoreactivity in reactive astrocytes has also been described in Alzheimer disease (Zhang et al., 1994; Minami et al., 1995) and, limited to demyelinating plaques, in multiple sclerosis (D'haeseleer et al., 2013; Hammond et al., 2014). Importantly, astrocyte-derived ET-1 prevents oligodendrocyte lineage progression from the OPC to the pre-myelinating stage without affecting cell proliferation, and inhibits OPC differentiation and remyelination after lysolecithin-induced focal demyelination in mice (Gadea et al., 2009; Hammond et al., 2014).

To investigate the possible functional consequences of ET-1 accumulation, we therefore assessed the numbers of pre-myelinating OPCs in the better preserved white matter areas of our patients, normal controls and sporadic SVD controls. Double labelling for the oligodendrocyte-specific marker olig2 and PDGFRα, an OPC marker, showed significantly higher OPC numbers in the patients than in sporadic SVD, and in both compared to the normal controls. The combination of increased OPC numbers and paucity of myelin indicates that OPC proliferate, but are inhibited from maturation. Our findings also suggest a positive correlation between the degree of astrocyte-derived ET-1 accumulation and OPC maturation block. Consistent with a greater inhibition of OPC maturation, the amounts of the mature myelin protein MBP were lower in the patients than in sporadic SVD. On these bases, we hypothesize that extended aberrant ET-1 expression contributes

to the vascular leukoencephalopathy of our patients via a combination of chronic hypoperfusion and stalled OPC differentiation.

The molecular mechanisms that trigger inhibition of OPC maturation in the context of ischemic white matter injury are likely to be multiple and related to factors both intrinsic and extrinsic to the OPCs. Importantly, many of these factors are neither age- nor disease-specific, suggesting that general mechanisms underlie inhibition of OPC maturation and remyelination in different types of chronic white matter injury. Astrocytes, either chronically reactive or abnormal, seem to play a central role. Expansion of the OPC pool in the setting of vascular brain injury of the elderly, for example, has also been associated with local accumulation of hyaluronan derived from reactive astrocytes, suggesting a hyaluronan-dependent OPC maturation arrest (Back et al., 2011). Of note, hyaluronan accumulation was also detected in the white matter of the two older ADLAVA patients in the context of another study (chapter 2.3). Diffuse white matter injury due to milder perinatal hypoxia-ischemia selectively triggers death of immature oligodendrocytes that are then rapidly regenerated from the pool of OPCs relatively more resistant to this kind of injury. Past the acute phase, the white matter becomes enriched in reactive glia, which generate inhibitory signals (including hyaluronan) that block OPC maturation into myelin-forming cells (Buser et al., 2012; Back & Rosenberg, 2014; chapter 2.3). The factors involved in OPC maturation arrest are also not disease-specific. Besides ischemic injury, hyaluronan and ET-1 both accumulate in multiple sclerosis lesions (Back et al., 2005; Sloane et al., 2010; D'haeseleer et al., 2013; Hammond et al., 2014), but also in VWM (chapter 2.3, unpublished observations).

In chapter 5.2, we describe the neuropathology of another genetic vascular disorder, Aicardi-Goutières syndrome (AGS). AGS is a genetically heterogeneous disorder characterized by CSF lymphocytosis and inappropriate induction of a type I interferon (IFN)-mediated immune response with increased intrathecal production of the IFN-type I cytokine IFN-α. In most, but not all, cases the condition manifests as a severe encephalopathy with a high associated morbidity and mortality (Aicardi & Goutières, 1984; Lebon et al., 1998; Crow, 2013a; Hofer & Campbell, 2013; Oda et al., 2014; Rice et al., 2014). Various lines of clinical and experimental evidence suggest that type I IFN has neurotoxic and neuroinflammatory effects on the central nervous system, especially during early neurological development, so that the raised levels of IFN-α seen in AGS patients most likely represent a primary pathogenic factor rather than an epiphenomenon (Crow et al., 2013b).

Imaging of AGS shows a leukoencephalopathy with intracranial calcifications and progressive brain atrophy. The distribution and extent of the calcification is variable, but the basal ganglia and deep white matter are usually affected (Crow, 2013a). Typical neuropathological findings include brain atrophy, lack of myelin, infarctions, and calcifications. Calcium deposits are found in areas of infarction or as small accretions not necessarily associated with necrosis (Goutieres et al., 1998; Kumar et al., 1998; Barth et al., 1999), however their source is still unknown. The findings of wedge-shaped infarcts, inhomogeneous loss of myelin and presence of calcifications also in the wall of small vessels and perivascular spaces has led to the suggestion that AGS represents a primary microangiopathy (Barth et al., 1999).

Neuropathology of our patient confirmed the extensive involvement of intraparenchymal small vessels and also showed multiple cortical infarctions often coincident with thrombotic obliteration of the leptomeningeal vessels. Microscopically, extensive calcification was widespread in the cerebral white matter, striatum and cerebellum. Of note, most calcium deposits were intramural or strictly associated with blood vessels. The calcified vessels showed loss of smooth muscle actin-immunoreactivity in smooth muscle cells, indicating loss of the normal contracting phenotype. These observations suggest that, in analogy with e.g. the cardiovascular system (Chen & Moe, 2012), calcific deposits in AGS arise in the smooth muscle cells of the vascular tunica media following de-differentiation to an osteoblast/chondrocytic phenotype. We then questioned if a relation exists between vascular calcification and IFN-α. Indeed, at concentrations comparable to what found patients' cerebrospinal fluid (CSF), IFN-α enhanced calcification of cultured human vascular smooth muscle cells *in vitro*. Notably, astrocytes are the cell type responsible for the excessive IFN-α production in brains of AGS patients (van Heteren et al., 2008), and transgenic mice with chronic astrocyte-specific overproduction of IFN-α develop a progressive leukoencephalopathy closely recapitulating AGS, including the vascular changes and calcifications (Akwa et al., 1998; Campbell et al., 1999). IFN-α, however, is likely not the only factor contributing to the calcifying microangiopathy typical of AGS. Other proinflammatory cytokines increased in AGS patients, including tumor necrosis factor-alpha (TNF-α) (Takanohashi et al., 2013), are able to promote *in vitro* calcification of vascular smooth muscle cells (Tintut et al., 2000). Notably, astrocytes are also a source of TNF-α (Lieberman et al., 1989). Overall, these results strongly support the concept of astrocytes and astrocyte-derived cytokines

as major contributors to the pathogenesis of the calcifying microangiopathy typical of AGS.

Brain MRI of AGS patients shows symmetric signal changes involving the deep white matter with relative sparing of the strictly periventricular area, corpus callosum, capsules and optic radiations (Crow, 2013a). Notably in many patients, as the one here described who died at 17 years of age, sequential MRIs reveal a progressive attenuation of the white matter abnormalities over time (chapter 5.2, personal observation). The non-necrotic white matter areas of our patient showed mild lack of myelin, reactive astrocytosis and little signs of inflammation. There were no myelin-laden macrophages, excluding ongoing demyelination. Compared to age-matched controls, we also found a mild loss of total oligodendrocytes with numerous caspase 3-positive apoptotic cells; however, OPC numbers were markedly increased compared to control white matter. The exact pathogenesis of the leukoencephalopathy in AGS is not known. Chronic hypoperfusion due to the microangiopathy and to a possible reduced angiogenesis (Akwa et al., 1998) could be a factor, in analogy with other types of ischemic injury to the white matter as illustrated above. Overexpression in CSF lymphocytes of cathepsin D, a (caspase 3-dependent) pro-apoptotic factor with myelin-directed protease activity induced by IFN-α, has also been evoked as a factor contributing to the myelin pathology (Izotti et al., 2008; Pulliero et al., 2013). Interestingly, CSF lymphocyte numbers progressively return to normal over time and the ratio between cathepsin D and its inhibitor cystatin F in AGS lymphocytes progressively diminishes (Pulliero et al., 2013). This suggests mechanisms other that lymphocytic neurotoxicity in maintaining oligodendrocytic pathology in AGS. The combination of mild oligodendropenia with increased OPC numbers and only mild lack of myelin found in our 17-year-old patient suggests a valid turnover of the oligodendrocyte lineage population with maturation of the precursors into myelin-forming cells, and highlights a remyelination potential which could account for the attenuation of the white matter signal changes observed on MRI in older AGS patients. Analysis of white matter oligodendrocytes in AGS patients of different ages and with different degrees in CSF INF-α could help understand if IFN-α also directly impacts on OPC differentiation.

The repair potential of genetic white matter disorders

One of the aims of this thesis was to define if a cellular pathology perspective applied to genetic white matter disorders may also be useful in assessing the white

matter repair potential and the obstacles to it. In facing the possibility to treat white matter disorders with factors enhancing endogenous repair and/or cell-based therapies, this information also needs to come into play.

The ability to repair injured tissue is fundamental to biology and central to survival. Evolutionary pressure has likely selected basic cellular and molecular responses to damage that are common across different tissues. Wound healing in skin has been used as a model system for dissecting repair mechanisms and provided insight into core cellular and molecular interactions (Gurtner et al., 2008). However, organ-specific features exist. Organ-specific cells specialized in inflammatory regulation and tissue repair are emerging as critical elements in organ-specific responses to insults. This applies particularly in the CNS where glial cells, which maintain the global cytoarchitecture and homeostatic regulation in the healthy brain, are also the principal responders to CNS insults. Physiological changes in glial cell functions during responses to insults may impact markedly on cellular interactions and CNS functions (Burda & Sofroniew, 2014). Dysfunctional glial cells as a result of a genetic defect have the additional potential to accumulate further layers of pathology causing growing injury and impeding repair.

Different types of injury to the CNS elicit different responses. Acute focal injuries trigger wound repair with tissue replacement, whereas chronic diffuse diseases often result in escalating tissue changes. Analysis of similarities and differences in such responses can provide valuable insights. Cellular responses to CNS insults involve complex interactions among cells of different lineages and functions, including CNS intrinsic neural and non-neural cells, and CNS extrinsic cells that enter from the circulation. The biology of cell types that participate in CNS responses to injury and disease models has generally been studied in isolation. There is increasing need to study interplay of different cells to understand mechanisms (Burda & Sofroniew, 2014).

One major aspect of endogenous repair in white matter disorders is remyelination. As illustrated in chapter 1, remyelination is a potentially highly effective process. Despite its efficiency in some clinical conditions, however, remyelination does not adequately occur in most genetic white matter disorders. The burden of the persistent lack of myelin is then borne by the axons, with neurodegeneration leading to increasing disability and disease progression (Franklin & ffrench-Constant, 2008).

Regardless of the perspective from which they are considered (clinical, biochemical, imaging or genetic), white matter disorders are highly complex and

heterogeneous diseases. The current pathological classification identifies different patterns of white matter damage depending on the presence or absence of myelin deposition, the modalities of myelin loss, and the presence of specific pathological features as intramyelinic edema. This classification, focused on the myelin, does not take into account the status of the oligodendrocytes. Throughout this thesis, we attempted an inventory of the oligodendrocyte lineage cells in different white matter disorders. We found that some diseases with lack of myelin are characterized by oligodendrocyte loss (chapter 4), whereas in others the oligodendrocyte lineage cells are preserved or even plentiful (chapters 2 and 5). A relation between oligodendrocyte numbers and remyelination has been noted in MS (Franklin & ffrench-Constant, 2008). According to one pathological classification, four lesion patterns can be identified in MS. In patterns I and II, dominated by T-lymphocytes and macrophages or characterized by a B-lymphocyte- or antibody-driven pathology, there is relative preservation of oligodendrocytes and remyelination may occur. In patterns III and IV, characterized by substantial oligodendrocyte loss and suggestive of primary oligodendrocyte pathology, remyelination is rare or absent. The correlation between the preservation of oligodendrocytes and remyelination suggests that one key factor determining the remyelination potential in MS and, we believe, in genetic white matter disorders is a disease pathogenesis in which oligodendrocytes are retained. However, as discussed in chapter 1, experimental data suggest that remyelination is mediated not by surviving oligodendrocytes but by their progenitors, the OPCs (Crawford et al., 2013; Franklin & Gallo, 2014). This implies that a critical and so far missing component to categorize white matter disorders is the status of the OPCs, which may inform on repair potential. For example, neuropathology of the analyzed child with AGS (chapter 5.2) showed increased OPC numbers and only mild paucity of myelin despite oligodendrocyte apoptotic loss, suggesting efficient remyelination. Information on the status of OPCs integrated in a better understanding of the mechanisms underlying failure of repair is also necessary to explore new therapeutic interventions, including the choice of (or the balance between) treatments supporting endogenous remyelination and exogenous cell-based therapies.

Our inventory of genetic white matter disorders shows paucity of oligodendrocyte lineage cells, including OPCs in hypomyelinating white matter disorders with oligopenia. Although OPCs are likely to respond to the lack/loss of myelin in primary hypomyelinating disorders as PMD, their intrinsic mutation may overcome the proliferation rate and render them ineffective in repair. The reasons for which oligodendrocytes and OPCs are scarce in secondary hypomyelination due to

neuronal/axonal pathology are also not clear. As summarized in chapter 1, electrical activity and proper axonal function are required for developmental myelination (Demerens et al., 1996; Wake et al., 2011). The synchronization between oligodendrocyte developmental programs and specific patterns of axonal activity is likely crucial for timing of oligodendrocyte differentiation and initiation of myelination. Compromised axonal function as expected in neurodegenerative polioencephalopathies could further impair the remyelination potential, as distribution of metabolites, organization of ionic channels and patterns of electrical activity, all necessary to support remyelination, are permanently altered (Franklin & ffrench-Constant, 2008). A different type of axonal-oligodendroglial communication has been recently characterized based on the demonstration of synapses on NG2-expressing OPCs (Bergles et al., 2000), which could shed some light on the pathogenesis of oligodendropenia in axonal pathologies. Under physiological conditions, axon collaterals in the white matter make direct glutamatergic and GABAergic synaptic contacts with OPCs (Lin & Bergles, 2003; Kukley et al., 2007; Ziskin et al., 2007). At early postnatal developmental stages, glutamatergic neuron-NG2 cell synaptic activity regulates proliferation and differentiation *in vivo* (Kukley et al., 2008; Ge et al., 2009; Mangin et al., 2012). Synapses on NG2 cells are then downregulated as OPCs mature into myelinating oligodendrocytes, indicating a specific role for these synapses at earlier developmental stages that precede myelination (Gallo et al., 2008; De Biase et al., 2010). Synaptic communication between neurons and OPCs likely also regulates oligodendrocyte regeneration and remyelination. Once they migrate from the subventricular zone in response to injury, NG2 cells receive glutamatergic synaptic inputs from axonal collaterals that are later downregulated at more mature stages during remyelination (Exteberria et al., 2010). Therefore, axons still establish and maintain contacts with NG2 cells during early phases of remyelination, suggesting that glutamate-induced depolarization contributes to NG2 cell cycle exit and initiation of differentiation also under pathological conditions. Failure of proper neuron-NG2 cell synaptic communication in hypomyelinating disorders could therefore contribute to impair OPC proliferation causing oligopenia, interfering with developmental myelination and, later, impeding remyelination.

Oligodendrocyte lineage cells and remyelination appear to be impaired in a different way in those white matter disorders with oligodendrocytosis. In these conditions (chapters 2.2-2.5 and 5.1), OPCs proliferate promptly in response to myelin injury, but fail to differentiate into myelin-forming cells. One explanation for this is that the chronically injured and demyelinated white matter contains factors

that hamper OPC differentiation. We could identify some potentially inhibiting factors, including hyaluronan and ET-1. Preliminary data suggest that additional inhibiting factors are also involved, including other ECM components (Harlow & Macklin, 2014). These findings are interesting in two respects. Firstly, as already illustrated, many of these inhibiting factors are not disease-specific. For example, an immunohistochemical survey of different white matter disorders form our tissue collection confirmed that, besides VWM, hyaluronan accumulates in ischemia-related white matter injury of infants and adults and chronic-active MS lesions (chapter 2.3; Back et al., 2005; Back et al., 2011; Buser et al., 2012). This suggests that hyaluronan-related inhibition of OPC differentiation is a general mechanism limiting developmental myelination and remyelination in the context of chronic white matter injury. As such, it could constitute a plausible therapeutic target for different disorders provided that the effects of any ongoing disease process on the new oligodendrocytes are suppressed. The same may hold true for ET-1 (chapter 5.1). Interestingly, for reasons we cannot explain, we found no immunohistochemical evidence of hyaluronan accumulation in genetic disorders with oligodendropenia, including PMD and infantile-onset AxD, MLD and Krabbe disease (chapter 2.3; Woodward et al., 2008; Faust et al., 2010; Tian et al., 2010). The survey of ET-1 expression in these disorders is still ongoing.

Another interesting aspect is that many of the factors inhibiting OPC differentiation are (largely or exclusively) derived from astrocytes. Astrocytes regulate developmental myelination and myelin maintenance in the adult brain (chapter 1) and are key players in the multicellular responses of the CNS to injury (Sofroniew, 2009; Burda & Sofroniew, 2014). Loss-of-function studies demonstrate that reactive gliosis exerts essential beneficial functions without which tissue damage and function would increase and repair would not occur (Sofroniew & Vinters, 2010; Burda & Sofroniew, 2014). With respect to myelin injury and repair, astrocytes may play both promoting and inhibiting roles on OPC proliferation and differentiation (Moore et al., 2011; Jones & Bouvier, 2014). Astrocytes impact ECM components, which, in turn, can modulate the phenotype of astrocytes resulting in (dys)regulation of astrocytic responses to injury also with respect to remyelination (Claycomb et al., 2013).

In general, astrocytes are increasingly recognized as active participants in the pathogenesis of many white matter disorders, beyond those due to defects in astrocyte-specific gene products. A current challenge in the field is to dissect the pathological aspects result from gain of abnormal astrocytic functions from those that are consequence of loss of beneficial functions. In this, VWM represents a

compendium of both aspects and an extreme example of failure of white matter repair. The consequences of VWM astrocytic immaturity and aberrant intermediate filament composition are still elusive, but a general negative impact on cell functions and on the efficiency of reactive gliosis can be anticipated. VWM astrocytes appear unable to contain injury and negatively impact ECM and tissue remodeling. Deficient and/or deeply perturbed astrocytic functions in VWM likely account for the loss of tissue observed in the more severely affected white matter areas. In this respect a role for hyaluronan, which also regulates astrocyte proliferation and maturation and inhibits glial scarring (Hou et al., 2005; Struve et al., 2005; Lin et al., 2009; Khaing et al., 2011), could be envisioned as well.

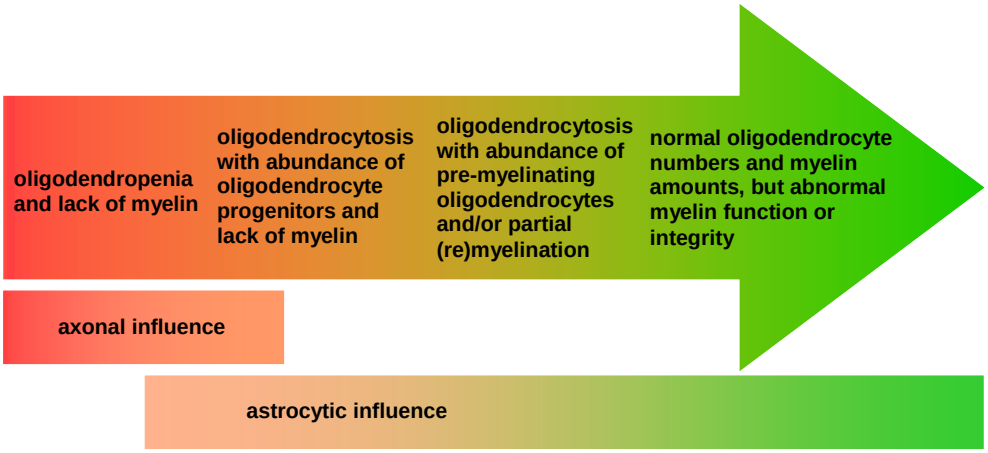

Figure 2. Inventory of the status of oligodendrocyte lineage cells in genetic white matter disorders in relation to the extra-oligodendrocyte cell types contributing to myelin degeneration, and to the repair/(re)myelination potential. Color code: red, inhibitory; green, permissive.

A new classification of genetic white matter disorders

Every classification reflects the knowledge of its time. The chronologic history of the classification of white matter disorders illustrates its repeated modifications.

Since the first attempt of categorization almost one century ago, white matter disorders have been classified according to their time at onset and disease course

(Bielschowsky & Henneberg, 1928); the type of demyelination, distinguishing endogenous from exogenous and specific from non-specific forms (Hallervorden, 1940); the morphologic and histochemical features of the tissue (Blackwood, 1957); the underlying biochemical defect integrated with clinical and histological data (Raine, 1984); and the biochemical group of compounds the metabolism of which is perturbed (Poser, 1987). More recent classifications follow miscellaneous criteria, including the organelle and/or the metabolism involved, the presence of a structural myelin defect and, in acquired disorders, the aetiologic category (van der Knaap & Valk, 2005). Classifications solely based on MRI are also employed (Schiffmann & van der Knaap, 2009).

The pathologic classification of white matter disorders referred to in chapter 1 recognizes four categories: hypomyelinating, demyelinating, dysmyelinating and myelinolytic. This classification has the major value of categorizing white matter disorders according to the main mechanism of white matter injury and recognizing the possibility that different pathomechanisms may contribute to determine a single disease. Based on what illustrated and discussed in this thesis, however, we question the choice of terms arguing that, also at the light of more recent pathogenetic insights, their reflection of the different disease categories is no longer tenable.

We therefore take advantage of this thesis to put forward a new classification of genetic white matter disorders that better reflects the scientific knowledge of this time (table 1). The contribution of cell types other than oligodendrocytes driving myelin dysfunction or degeneration, including astrocytes and neurons, is considered also to provide additional information as to the pathogenesis and the repair potential. Given the pathogenetic complexity of many white matter disorders, the classification recognizes the possibility that a specific disease does not primarily affect one cell type only and with that belongs to more than one category.

We propose to classify white matter disorders into four main categories.

A first category of "**myelin disorders**" includes those disorders in which oligodendrocyte and myelin are primarily or predominantly affected. These are the hypomyelinating disorders, the demyelinating disorders ("leukodystrophies"), and the diseases with myelin vacuolization, either primary defects in ion-water homeostasis or secondary defects due to toxic myelin edema.

A second category comprises white matter disorders due to defects in astrocyte-specific gene products. We would name this category "**astrocytopathies**".

A third category encompasses white matter disorders secondary to neuronal or axonal defects. We adopt the term “**leuko-axonopathies**” for this category, to highlight that the white matter degeneration results from an abnormal axo-glia interaction. The term was originally proposed by John K. Fink (University of Michigan and Geriatric Research Education and Clinical Center, Ann Arbor Veterans Affairs Medical Center, Ann Arbor, MI).

A fourth category includes cases of **non-selective white matter degeneration**, due to pathomechanisms not included in the other categories, or of which the pathogenesis is still obscure.

Not all white matter disorders that can be currently diagnosed have been pathologically characterized. For this reason, the assignment of a certain condition to one or the other category also depends on data derived from imaging studies and, when known, on the supposed function of the associated mutated protein. For some white matter disorders, including Sjögren-Larsson syndrome, adult onset autosomal dominant leukodystrophy, sialic acid storage diseases and Lowe syndrome, the cellular pathomechanisms are presently still so unclear that proper classification is not possible.

Given that the field of white matter biology and white matter disorders evolves at a tremendous speed, we expect this classification to be provisional and look forward to the day it will be challenged by new knowledge.

Table 1. A new classification of genetic white matter disorders

Myelin disorders

Hypomyelinating

a. Pelizaeus-Merzbacher disease
b. peripheral neuropathy, central hypomyelination and Waardenburg-Hirschsprung syndrome
c. Cx47-related Pelizaeus-Merzbacher-like disease

Demyelinating

a. metachromatic leukodystrophy
b. multiple sulfatase deficiency
c. globoid cell leukodystrophy (Krabbe disease)
d. X-linked adrenoleukodystrophy
e. Zellweger spectrum disorders
f. Refsum disease

Myelin vacuolization

a. primary defects in ion-water homeostasis
    1. megalencephalic leukoencephalopathy with subcortical cysts
    2. ClC2-related disease
    3. Cx32-related (X-linked) Charcot-Marie-Tooth disease
    4. congenital muscular dystrophies (?)
    5. Cx47-related Pelizaeus-Merzbacher-like disease (?)
b. secondary defects in ion-water homeostasis due to toxic myelin vacuolization
    1. mitochondrial diseases with leukoencephalopathy
        - MELAS
        - Kearns Sayre syndrome
        - MNGIE
        - Leigh syndrome and mitochondrial leukoencephalopathies
        - pyruvate carboxylase deficiency
        - pyruvate dehydrogenase deficiency
        - *APOPT1*-related cavitating leukodystrophy
    2. phenylketonuria
    3. maple syrup urine disease
    4. Canavan disease
    5. glycine leukoencephalopathy and variants
    6. glutaric aciduria type I
    7. propionic acidemia
    8. L-2-hydroxyglutaric aciduria
    9. D-2-hydroxyglutaric aciduria
    10. hyperhomocysteinemia
    11. galactosemia

Table 1 (cont.). A new classification of genetic white matter disorders

12. urea cycle disorders
13. nonketotic hyperglycinemia
14. Wilson disease
15. Menkes disease

Astrocytopathies

a. Alexander disease
b. megalencephalic leukoencephalopathy with subcortical cysts
c. vanishing white matter
d. Aicardi-Goutières syndrome and variants
e. giant axonal neuropathy type I
f. adult polyglucosan body disease
g. Wilson disease
h. Menkes disease
i. congenital muscular dystrophies
j. fragile X premutation
k. ADLAVA

Leuko-axonopathies

a. hypomyelination with atrophy of the basal ganglia and cerebellum
b. hypomyelination with congenital cataract
c. *CSF1R*-related disorders
    1. hereditary diffuse leukoencephalopathy with spheroids
    2. pigmentary ortochromatic leukodystrophy
d. early onset neuronal degenerative disorders
    1. gangliosidosis GM1 and GM2
    2. infantile neuronal ceroid lipofuscinosis
    3. *AGC1*-related disease
    4. *AIMP1*-related diseases
    5. *HSPD1*-related disease
    6. fucosidosis
    7. serine synthesis defects
e. Pol III-related leukodystrophies
f. adrenomyeloneuropathy
g. Zellweger spectrum disorders
h. mucopolysaccharidoses
i. cerebrotendineous xanthomatosis
j. Cockayne syndrome

Table 1 (cont.). A new classification of genetic white matter disorders

k. Fragile X premutation
l. *AARS2*-related leukoencephalopathy
m. leukoencephalopathy with brainstem and spinal cord involvement and high lactate
n. hypomyelination with brainstem and spinal cord involvement and leg spasticity
o. Nasu-Hakola disease
p. dentatrubropallidoluysial atrophy

Nonselective white matter degeneration

a. Hypoxia-ischemia
    1. CADASIL
    2. CARASIL
    3. cerebral amyloid angiopathy
    4. ADLAVA
    5. Aicardi-Goutières syndrome and variants
    6. Labrune disease
b. Fabry disease
c. Nasu-Hakola disease

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Acknowledgements

A special thank goes to Marjo van der Knaap, who co-parented the idea of approaching white matter disorders from a cellular pathology perspective. I am grateful for these years spent in the Center for childhood white matter disorders, the opportunity to integrate basic science into my background, all the support and the future plans we now share.

I thank Marjo, Paul van der Valk and Jaap Valk for the chance of joining the departments of Child Neurology and Pathology of the VU University medical center. I am grateful to Paul, Annemieke Rozemuller, Pieter Wesseling, Patrick van der Voorn and Wouter Kamphorst for their warm welcome into the neuropathology club of the VUmc, and to Marjo, Paul and Ellis Barbé for the opportunity to be part --at least for some time- of this club. A special thank goes to Jim Powers for his dedication in teaching and supervising me on the study of the neuropathology of defined and undefined white matter disorders.

I am obliged to Ilja Boor for the years we spent in the lab, the fruitful collaboration and charming friendship that stemmed from our work together. I thank Nienke Postma for her friendly and witty teamwork in the everyday life in the lab. I am grateful to Truus Abbink, Nicole Wolf, Liane Klok and Vivi Heine for the teeming scientific discussions and present and future work together, and to Gert Scheper for his supervision during the first period of my PhD. I thank Mohit Dubey, Sietske Kevelam and Stephanie Dooves for the amusing time spent preparing our manuscripts, all of which are part of this thesis. I am grateful to all past and present members of the Center for childhood white matter disorders for their hearty friendship and support.

This thesis also results from what I was before and am besides being a PhD student in the Center for childhood white matter disorders. I am indebted to Orso Bugiani and William Demyer for showing me how fascinating a multidisciplinary approach to the human brain may be. A special thank goes to the department of Pediatric neurology of the C. Besta institute, Milan, and especially to Graziella Uziel and Isabella Moroni, two friends from whom I learnt how to become a child neurologist. I am grateful to Massimo Zeviani and to Alberto Bizzi and Mario Savoiardo, also from the C. Besta institute, for their friendship and all they taught me about the genetics and neuroradiology of white matter disorders. I thank Viola Caretti, Esther Hulleman, Dannis van Vuurden, Sophie van Veldhuijzen-van Zanten and all the others in the VUmc team working on diffuse intrinsic pontine glioma, and Sandra Amor and the multiple sclerosis group for the opportunity of an

alternative look at glia biology and pathology and the extraordinary past and ongoing collaboration. I am grateful to Frietson Galis and Claartje ten Broek because our work on skeletal malformations at the edge with comparative anatomy inspired me to also apply an evo-devo approach to white matter diseases.

I thank the mortuary assistants of the VUmc for their skilful help in collecting the brain tissue on which large pat of this thesis is based, and the photographers of the department of Pathology.

I am grateful to my supporters Annemieke Rozemuller and Eleonora Aronica for their backup.

I am obliged to all the others I collaborated and work with, including the team of the Dutch brain bank, to whom I apologize for not mentioning them by name. I am also grateful to the Pathology residents and pathologists of the VUmc, and to the students I supervised in the lab, because interacting with them gives me the opportunity to learn something new everyday.

My final thank goes, of course, to my family: my dad Orso, my mom Niella, my sisters Martina and Melania, Jake, my niece and nephews Gabriel Jay, Harry and Nocciola, and Flavio. For backing me up, always.

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