Exploring Alzheimer Molecular Pathology in Down’s Syndrome Cerebrospinal Fluid

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Key Words
Down’s syndrome · Alzheimer’s disease · Orexin-A · Amyloid-β · YKL-40 · Tau

Abstract
Background: Individuals with Down’s syndrome (DS) develop early Alzheimer’s disease (AD) with β-amyloid (Aβ) plaque pathology. The extra amyloid precursor protein (APP) gene copy in DS is believed to result in a 50% increase in Aβ production, but it is unclear how this relates to the development of other AD hallmarks, including axonal degeneration and microglia cell activation, and to other neurological problems in DS, including disturbed sleep regulation. Objective: To evaluate if cerebrospinal fluid (CSF) biomarkers for cerebral amyloidosis, axonal degeneration, microglial activation and sleep regulation were altered in young and old patients with DS, and if these biomarkers were related to altered Aβ and APP metabolism, reflected by CSF levels of different Aβ and APP peptides. Methods: CSF from DS patients (n = 12) and healthy controls (n = 20) were analyzed for Aβ peptides (Aβ1–42, AβX–38/40/42), secreted APP species (sAPPα/β), biomarkers for AD-like axonal degeneration [total tau (T-tau), phosphorylated tau], microglial activation (YKL-40, CC chemokine ligand 2) and orexin-A, which is a peptide involved in sleep regulation. We compared biomarker levels between groups and tested for relations between biomarkers, disease stage and age. Results: Several of the markers were specifically increased in DS, including AβX–40, sAPPα and sAPPβ. Orexin-A was significantly decreased in DS and correlated with Aβ and sAPP. Orexin-A decreased with age in DS, while T-tau and YKL-40 increased with age. Conclusion: Down’s patients have increased APP and Aβ production and increased microglial activation with age. The orexin-A metabolism is disturbed in DS and may be linked to APP and Aβ production. Biomarker studies of DS may contribute to our understanding of the amyloidogenic and neurodegenerative process in AD.

Introduction

Down’s syndrome (DS), caused by trisomy 21, is one of the most common genetic birth defects and was first described by Down [1] in 1866. DS leads to the develop-
ment of amyloid-β (Aβ) plaque pathology, likely due to the extra copy of the amyloid precursor protein (APP) gene on chromosome 21. Aβ accumulation has been found in DS individuals from 8 to 12 years of age [2, 3]. Later in life, DS is associated with an increased risk of Alzheimer’s disease (AD) [4, 5], which is a dementing illness characterized by the presence of Aβ plaques, neurofibrillary tangles, axonal degeneration and microglial cell activation in the brain [6]. In addition, autopsy studies have found that nearly all DS adults over 35–40 years have both neurofibrillary tangles and plaques [7].

The localization of the APP gene to chromosome 21 [8–10] and the association of Aβ pathology with both AD and DS were important for the development of the amyloid cascade hypothesis, which states that a disturbance in the Aβ homeostasis is the causal factor in the neuronal degeneration characteristic of AD [11]. However, it is not known how developments of different disease hallmarks are linked to Aβ pathology in DS. Also, it is not known how Aβ pathology is related to other neurological problems in DS, including disturbed sleep regulation [12–14]. To gain insight into these molecular mechanisms, we tested if cerebrospinal fluid (CSF) biomarkers related to Aβ and APP metabolism (Aβ peptides and APP species), axonal injury [total tau (T-tau) and phosphorylated tau (P-tau)], microglial activation [chitinase-3-like protein 1 (YKL-40) and CC chemokine ligand 2 (CCL2)] and sleep regulation (orexin-A) differed between controls and DS patients. We also tested if biomarker levels differed across the age span in DS. We hypothesized that we would find biomarker evidence of increased Aβ42 production in younger DS patients, as well as AD-like alterations indicating Aβ plaque formation, axonal degeneration, plaque pathology and microglial activation in older DS patients.

The 42-amino acid Aβ variant Aβ1–42 is a useful biomarker to identify AD patients and study AD pathology [15, 16]. Previous studies have shown that young DS patients have higher CSF concentrations of Aβ1–42 than older DS patients [17] and that middle-aged DS patients have decreased Aβ1–42 compared to controls [18]. Thus, Aβ1–42 seems to be elevated in the early stages of DS and decrease with age, which may reflect development of plaque pathology with plaques acting as peptide sinks in the brain. We tested if we could replicate previous findings of increased CSF Aβ1–42 in young DS patients.

A variety of Aβ peptides are generated from the transmembrane APP protein through sequential cleavage by β- and γ-secretase [19]. APP is first cleaved by β-secretase at the N terminus, generating a soluble APP fragment (sAPPα) and the membrane-bound fragment C99, which can be further processed by γ-secretase into various Aβ peptides including Aβ1–38, Aβ1–40 and Aβ1–42 [20]. In the α-secretase pathway, α-secretase cleaves APP, generating sAPPα [21]. We extended previous DS studies by measuring several Aβ peptides (Aβ1–42, AβX–38/40/42) and sAPP species (sAPPα and sAPPβ) in young and old DS patients to test whether they reflect early disease development.

One previous study on DS patients showed a significant correlation between age and CSF tau, with lower concentrations of tau in the youngest individuals, suggesting that axonal degeneration increased in older patients [17]. Here, we measured CSF T-tau and P-tau across the age span in DS and tested correlations between these markers of AD-like axonal degeneration and markers of Aβ and APP metabolism.

Microglial activation has been suggested to play an important role as a mediator of Aβ toxicity in the development of AD [22], but to our knowledge no previous study has measured in vivo biomarkers of microglia activity in DS patients. Therefore, we measured the microglial activation markers YKL-40 [23] and CCL2 [24] to determine if they were altered in DS and if they correlated with Aβ or APP levels.

Finally, DS patients may present a variety of neurological symptoms, including sleep dysregulation [12–14], and it has not been studied previously if this is related to Aβ pathology. Recently, it was shown that the neuropeptide orexin, which has key functions in sleep regulation [25], may regulate Aβ dynamics, and it was proposed that it may play a role in the pathogenesis of AD [26, 27]. It has further been shown that there is a relationship between AD pathology and orexin. The final goal of this study was therefore to determine orexin-A levels in DS CSF and to test if orexin-A levels correlated with Aβ or APP levels.

Subjects and Methods

Subjects

Lumbar CSF from persons with DS (6 women and 6 men, mean age at lumbar puncture 41 ± 11 years) was collected in the Vaalijärvi Hospital in Piekäsmäki, Finland, as described before [17]. Lumbar CSF samples from age- and gender-matched control individuals from the Surgical Stress Study (8 women and 12 men, mean age at lumbar puncture 40 ± 15 years) were selected as the control group [28]. The study was approved by the local ethics committee of the Kuopio University Hospital and the ethics committee of the University of Gothenburg. Written informed consent was obtained from all participants included in the control group, and detailed information about the study was given to the participants and their caregivers and written informed consent for participation in the study was obtained from them.

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Alzheimer’s Disease Biomarkers in Down’s Syndrome
**Immunassays against Aβ Species, sAPPα/β, T-Tau and P-Tau**

CSF was analyzed for AβX–38, AβX–40, AβX–42, sAPPα and sAPPβ [limit of quantification: 8.2, 27.4, 8.2, 31.2 and 31.2 pg/ml, respectively; intra-assay coefficient of variation (CV): 4, 7, 1, 4 and 1%, respectively] using the Meso Scale Discovery (MSD) Aβ Triplex assay for the Aβ peptides and the MSD sAPPα/sAPPβ multiplex assay for the sAPP domains as described by the manufacturer (MSD, Gaithersburg, Md., USA).

The CSF T-tau concentration was determined using a sandwich ELISA [INNOTEST hTAU Ag, Innogenetics, Ghent, Belgium] specifically constructed to measure all tau isoforms irrespective of phosphorylation status, as previously described [intra-assay CV 7%] [29]. Tau phosphorylated at threonine 181 was measured using a sandwich ELISA method [INNOTEST PHOSPHO-TAU(181P), Innogenetics], as previously described [intra-assay CV 2%] [30]. CSF Aβ1–42 levels were determined using a sandwich ELISA [INNOTEST β-AMYLOID(1–42), Innogenetics] specifically constructed to measure Aβ containing both the 1st and 42nd amino acid, as previously described [intra-assay CV 7%] [31]. In this assay, the monoclonal antibody 21F12, which is highly specific for the C terminus of Aβ42, was used for capture, and 5D6, which is specific to the N terminus, was used as detector.

**Microglial Activation Markers**

CSF levels of YKL-40 [limit of quantification 6,250 pg/ml, intra-assay CV 5%] were analyzed with a commercial ELISA (R&D Systems, Minneapolis, Minn., USA), and levels of CCL2 [limit of quantification 0.61 pg/ml, intra-assay CV 14%] were measured with the MSD human MCP-1 Ultra-Sensitive Kit (MSD). Both markers were analyzed as described by the manufacturer.

**Orexin-A**

Orexin-A was measured using an in-house RIA. In brief, 50 μl of CSF from control and DS samples were diluted with 200 μl of assay buffer (0.01 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl, 0.3% bovine serum albumin, 0.1% Triton X-100 and 0.001 M EDTA). Calibrators (Orexin-A human PolyPeptide Sc 1337) and diluted samples (100 μl each) were incubated with 100 μl of orexin-A rabbit antiserum (Sigma O9756), final dilution 1/100, in 0.1 M NaCl, 0.3% bovine serum albumin, 0.1% Triton X-100 and 0.001 M EDTA. Calibrators (Orexin-A human PolyPeptide Sc 1337) and diluted samples (100 μl each) were incubated with 100 μl of orexin-A rabbit antiserum (Sigma O9756), final dilution 1/350,000 in assay buffer, at 2–8 °C for 24 h.

A second incubation was performed in the same manner or at 2–8 °C for 24 h after addition of 100 μl of 125I-orexin-A (diluted to 0.01 μCi/ml, 100 μl) to each sample. Radioactive ligand (125I-orexin-A) was synthesized using a modified chloramine-T method [32], purified with reverse-phase HPLC using a μ-Bondapak C18 column (3.9 × 300 mm, 125 Å, 10 μm, Waters Code Mo. 27324), diluted 1/10 in CH3CN and stored at −20 °C until use.

Free and bound tracers were separated by adding 200 μl of 2% normal rabbit serum (Sigma R9133; in 0.01 M sodium phosphate buffer, pH 7.4), 100 μl of goat anti-rabbit IgG (GAK Sigma R0881; in sodium phosphate buffer, pH 7.4) and 500 μl of 6% polyethylene glycol solution (molecular weight 6,000; in sodium phosphate buffer, pH 7.4) before incubation (21 °C, 10 min) and centrifugation (1,500 g, 21 °C, 10 min).

The supernatant was discarded and the precipitate measured in an automatic gamma counter (Wizard 1470; counting efficiency 75%) connected to an immunoassay software program (MultiCalc Advanced, Wallac Oy, Finland).

The intra-assay CV and interassay CV for CSF samples at 320 and 801 pg/ml were both <6%. The RIA was performed with duplicates of calibrators, controls and samples.

**Statistical Methods**

Due to nonnormal distributions for some of the analytes, the group comparisons were made using the nonparametric Mann-Whitney U test, and correlations are presented as Spearman’s ρ. Correlations with age were tested by using age both as a continuous and binary variable (with groups dichotomized into subjects younger and older than 40 years). Prism 6 (GraphPad Software, La Jolla, Calif., USA) was used for all statistical analyses. Due to the discovery nature of this study, p values were not corrected for multiple comparisons. The results were considered significant if p < 0.05 (two-tailed).

**Results**

**CSF Levels of Biomarkers in DS Patients and Controls**

We initially compared biomarker levels between DS patients and controls. The concentrations of AβX–40, sAPPα and sAPPβ were significantly increased (p = 0.048, 0.015 and 0.036, respectively) in DS patients, while AβX–38, AβX–42 and Aβ1–42 were similar across the two groups (fig. 1a–f). The axonal damage markers T-tau and P-tau (fig. 1g, h) and the microglia activity markers CCL2 and YKL-40 were also similar between the two groups (fig. 1i, j). The levels of orexin-A were reduced in DS patients (p = 0.0007; fig. 1k). Table 1 shows the demographic and biomarker characteristics of DS patients and controls.

**Correlation of Biomarkers with Age**

In the DS group, the concentration of Aβ1–42 correlated negatively with age (r = −0.69, p = 0.015), while T-tau had a positive correlation with age (r = 0.68, p = 0.025; fig. 2a). YKL-40 correlated positively with age for both DS patients (r = 0.76, p = 0.0062) and controls (r = 0.50, p = 0.024; fig. 2a). None of the other markers correlated with age. In figure 3, scatterplots show the distribution of the markers.

Next we compared biomarker levels between old (>40 years) and young (<40 years) subjects. For the controls (age ≤40 years, n = 13; age >40 years, n = 7), no statistical differences were observed between the groups. However, older DS patients (age ≤40 years, n = 6; age >40 years, n = 6) had significantly increased levels of T-tau, P-tau and YKL-40 compared to younger DS patients (p = 0.0087, 0.046 and 0.015, respectively; fig. 1g–i). None of the other markers differed between young and old DS patients.
Correlations of Microglial Activation Markers with Other Biomarkers

The concentrations of YKL-40 measured in DS patients had a significant correlation with T-tau (r_s = 0.84, p = 0.0022) and P-tau (r_s = 0.74, p = 0.0082; fig. 2b), while CCL2 did not correlate with either of these markers (fig. 2c). There were no correlations of microglia markers with other biomarkers in controls.

Correlations of Orexin-A with Other Biomarkers

We found a significant correlation between orexin-A and AβX–38 and AβX–40 in both controls (r_s = 0.78 and 0.58, respectively) and DS patients (r_s = 0.85 and 0.78, respectively), while AβX–42 only correlated with orexin-A in the control group (r_s = 0.56; fig. 2d). We also found a strong correlation of orexin-A with sAPPα, SAPPβ and T-tau in the DS group (r_s = 0.81, 0.87 and 0.72, respectively) but not in the controls.
**Discussion**

This is the first study systematically testing multiple CSF biomarkers for Aβ pathology, axonal degeneration, microglial activity and sleep dysregulation in patients with DS. The major findings were that (1) levels of AβX–40, sAPPα and sAPPβ were significantly increased in DS patients compared to controls; (2) Aβ1–42 decreased with age, and T-tau and P-tau increased with age in DS patients, while the microglia marker YKL-40 increased with age in both DS patients and controls; (3) YKL-40 levels were increased in DS patients with signs of AD-like axonal injury, and (4) the sleep-regulating peptide orexin-A was significantly reduced in DS patients compared to controls and was related to CSF levels of several Aβ and sAPP peptides in both DS patients and controls, as well as to signs of axonal injury in DS patients. Taken together, these results support the amyloid cascade hypothesis.

**Table 1.** Demographic and biomarker characteristics of DS patients and controls

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Controls (n = 20)</th>
<th>DS patients (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female/male (% female)</td>
<td>8/12 (33%)</td>
<td>6/6 (50%)</td>
</tr>
<tr>
<td>Age at LP, years</td>
<td>40±15</td>
<td>41±11</td>
</tr>
<tr>
<td>AβX–38, pg/ml</td>
<td>1,295±359</td>
<td>1,892±1046</td>
</tr>
<tr>
<td>AβX–40, pg/ml</td>
<td>9,014±2,036</td>
<td>13,583±7,669</td>
</tr>
<tr>
<td>AβX–42, pg/ml</td>
<td>1,147±280</td>
<td>1,300±572</td>
</tr>
<tr>
<td>Aβ1–42, pg/ml</td>
<td>674±145</td>
<td>637±201</td>
</tr>
<tr>
<td>sAPPα, pg/ml</td>
<td>496±216</td>
<td>718±242</td>
</tr>
<tr>
<td>sAPPβ, pg/ml</td>
<td>362±140</td>
<td>512±189</td>
</tr>
<tr>
<td>T-tau, pg/ml</td>
<td>210±87</td>
<td>431±369</td>
</tr>
<tr>
<td>P-tau, pg/ml</td>
<td>34±8.8</td>
<td>52±31</td>
</tr>
<tr>
<td>CCL2, pg/ml</td>
<td>457±114</td>
<td>519±196</td>
</tr>
<tr>
<td>YKL-40, pg/ml</td>
<td>89,648±55,000</td>
<td>97,381±50,202</td>
</tr>
<tr>
<td>Orexin-A, pg/ml</td>
<td>716±146</td>
<td>488±178</td>
</tr>
</tbody>
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Values represent means ± SD. LP = Lumbar puncture.
by confirming that persons with DS have a lifelong over-production of APP, while plaque pathology, axonal degeneration and microglia activation is present mainly in older subjects. The results also indicate dramatic effects of DS on the sleep-regulating peptide orexin-A and suggest that this is related to Aβ metabolism and axonal degeneration.

The finding that levels of AβX–40, sAPPα and sAPPβ were significantly increased in DS patients compared to controls agrees with an overproduction of APP due to the extra copy of chromosome 21 in DS. Furthermore, CSF Aβ1–42 levels correlated inversely with age in DS, indicating late-life Aβ plaque pathology development, replicating findings from an earlier study [17]. A study on carriers of genetic mutations causing autosomal dominant AD found increased CSF Aβ1–42 in subjects about 2 decades before expected symptom onset [33]. This is a finding we could not replicate; there was considerable overlap for CSF AβX–42 and CSF Aβ1–42 between young (<40 years) DS patients and controls (fig. 2c, d, grey bars). It is possible that an investigation directed specifically at very young patients and controls

Fig. 3. Scatter plots displaying the distributions of the markers with p < 0.05. Open circles represent DS patients, and grey circles represent healthy controls.
(in their 20s) could identify elevated CSF Aβ42 in very young DS patients. None of the other APP-related markers correlated with age, suggesting that levels of these markers are unaffected by the presence of Aβ pathology. The microtubule-associated protein T-tau showed a significant correlation with age, with clearly increased levels in the older DS individuals, for both T-tau and P-tau, resembling the evolution of biomarker changes in AD [34].

Microglial activation has been associated with AD, and it has been suggested that microglia and astrocytes contribute to AD progression [35, 36]. Recently, it was shown that the microglial activation marker YKL-40 was elevated in AD compared to controls [37, 38]. Here, we found a correlation between the levels of YKL-40 and age in both DS and control individuals, with clearly increased levels in the older DS patients. Relatively little is known about inflammation and microglial activation in the DS brain, but there are a number of different inflammatory genes on chromosome 21 which may induce neuroinflammation in DS [39]. YKL-40 also correlated with T-tau and P-tau in DS patients, indicating an association between neuronal damage and microglial activation. Our findings support the presence of microglial activation in DS patients with AD-like neuronal injury and support the use of YKL-40 as a readout for this phenomenon in clinical research [38].

The second inflammatory marker studied here, CCL2, has been suggested to be a predictive indicator for the appearance or severity of AD. However, CCL2 results in AD are conflicting; one study showed increased CSF levels in AD [40], but another could not replicate this finding [41]. We could not find any association between CCL2 and age, or differences between DS and controls. Further, there was no correlation between YKL-40 and CCL2 (fig. 2b). The different associations for YKL-40 and CCL2 are consistent with a previous report from our laboratory [41], where YKL-40 and CCL2 did not correlate in healthy controls or AD patients, although they correlated in patients with other dementias. There are several possible explanations for these differences in associations. For example, it is possible that CSF levels of YKL-40 and CCL2 do not only represent brain microglial activity but also other pathological processes, which may be differently regulated in different conditions. Alternatively, the release of YKL-40 and CCL2 may depend on specific states of microglia activity, or there may be differences in their biological turnover time, distorting the associations. However, we suggest that the present data, together with previous publications, argue against the use of CCL2 to monitor microglial activation in neurodegenerative diseases.

To our knowledge, orexin-A, which is a key molecule in sleep regulation [42, 43], has never been measured previously in DS patients. Its association with sleep regulation and narcolepsy is well established, and it has also been implicated in other neurological disorders [for a review, see 44]. Information regarding sleep was not available for the subjects included in the study, but sleep problems in DS are common and well described [12–14]. Our finding that DS patients have reduced CSF orexin-A levels provides novel evidence of altered orexin-A regulation in DS. Furthermore, we found that orexin-A correlated with AβX–38/40/42 in both DS patients and control individuals, and with sAPPα and sAPPβ in DS, but not with Aβ1–42 in either group. These findings confirm and add to previous studies describing associations between Aβ and sleep regulation [45]. A few studies have examined associations between orexin-A and Aβ, with somewhat conflicting results. One study in rodents showed that the levels of Aβ in brain interstitial fluid correlated with wakefulness and increased significantly during acute sleep deprivation and during orexin-A infusion [26]. Another study showed that the levels of orexin-A in postmortem ventricular CSF was lower in AD patients compared to controls [46]. In contrast, one study found increased levels of CSF orexin-A in (female) AD patients but no associations between Aβ1–42 and orexin-A in CSF [47]. The correlations identified in this study between CSF orexin-A and several different Aβ and sAPP peptides suggest that orexin-A expression is coupled to APP production and/or degradation but not to the presence of amyloid plaques.

A limitation of this study was the large number of statistical tests performed, increasing the risk of type I statistical errors. Due to the discovery nature of the study, we did not correct for multiple comparisons. However, for most key findings, multiple consistent associations were identified, supporting the main conclusions. The results of this discovery study should be validated in independent cohorts.

In summary, we found evidence of increased Aβ production at an early age in DS and development of Aβ plaque pathology later in life. We also found biomarker evidence of microglial activation in DS, and this activation correlated with the presence of AD-like axonal injury. Finally, we found reduced levels of the sleep-regulating peptide orexin-A in DS and evidence that this protein may be coordinated with APP metabolism. These findings from DS may increase our understanding of disease processes leading to AD.
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