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Review

# Search for biomarkers of Alzheimer's disease: Recent insights, current challenges and future prospects☆

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ABSTRACT

Due to the trend of prolonged lifespan leading to higher incidence of age-related diseases, the demand for reliable biomarkers of dementia rises. In this review, we present novel biomarkers of high potential, especially those found in blood, urine or saliva, which could lead to a more comfortable patient experience and better time- and cost-effectivity, compared to the currently used diagnostic methods. We focus on biomarkers that might allow for the detection of Alzheimer's disease before its clinical manifestations. Such biomarkers might be helpful for better understanding the etiology of the disease and identifying its risk factors. Moreover, it could be a base for developing new treatment or at least help to prolong the presymptomatic stage in patients suffering from Alzheimer's disease. As potential candidates, we present, for instance, neurofilament light in both cerebrospinal fluid and blood plasma or amyloid β in plasma. Above all, we provide an overview of different approaches to the diagnostics, analyzing patient's biofluids as a whole using molecular spectroscopy. Infrared and Raman spectroscopy and especially chiroptical methods provide information not only on the chemical composition, but also on molecular structure. Therefore, these techniques are promising for the diagnostics of Alzheimer's disease, as the accumulation of amyloid β in abnormal conformation is one of the hallmarks of this disease.

## List of abbreviations

| | |
|---|---|
| 8-OHdG | 8-hydroxy-2′-deoxyguanosine |
| Aβ | amyloid β peptide |
| Aβo | amyloid β peptide oligomers |
| $Aβ_{42}$ | 42-amino acid form of amyloid β peptide |
| $Aβ_{40}$ | 40-amino acid form of amyloid β peptide |
| AD | Alzheimer's disease |
| ADRDA | Alzheimer's Disease and Related Disorders Association |
| AChE | acetylcholinesterase |
| ATR | attenuated total reflection |
| AUC | area under the curve |
| BACE-1 | β-site amyloid precursor protein cleaving enzyme 1 |
| BBB | blood-brain barrier |
| BDNF | brain-derived neurotrophic factor |
| CD | circular dichroism |
| CNS | central nervous system |
| CSF | cerebrospinal fluid |
| ECD | electronic circular dichroism |
| ELISA | enzyme-linked immunosorbent assay |
| FDG | fluorodeoxyglucose |
| FTIR | Fourier-transformed infrared |
| GFAP | glial fibrillary acidic protein |
| HDL | high-density lipoprotein |
| IR | infrared |
| LC | liquid chromatography |
| LDA | linear discriminant analysis |
| MALDI-TOF MS | matrix-assisted laser desorption/ionization time-of-flight mass spectrometry |
| MCI | mild cognitive impairment |
| MCP-1 | monocyte chemoattractant protein-1 |
| MRI | magnetic resonance imaging |
| MS | mass spectrometry |
| NFL | neurofilament light chain |
| NINCDS | American National Institute of Neurological and Communicative Disorders |
| NIR | near infrared |
| NMR | nuclear magnetic resonance |
| NPV | negative predictive value |
| NSE | neuron-specific enolase |
| PCA | principal component analysis |
| PET | positron emission tomography |

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| | |
|---|---|
| PiB | Pittsburgh compound B |
| PPV | positive predictive value |
| pTau | phosphorylated tau protein |
| QDA | quadratic discriminant analysis |
| ROA | Raman optical activity |
| sNRG-1 | soluble neuregulin-1 |
| SUVR | standardized uptake value ratio |
| TNFR-1 | tumor necrosis factor receptor 1 |
| tTau | total tau protein |
| VCD | vibrational circular dichroism |
| VLDL | very low-density lipoprotein |
| VOA | vibrational optical activity |
| WBCs | white blood cells |

## 1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease and the leading cause of dementia, representing approximately 60 to 80% of all cases [1]. Although AD was first described more than a century ago [2], the cause and early pathogenesis thereof has not yet been fully understood and a proper treatment does not exist. Over the years, several hypotheses about AD origin have been formulated [3–8]. The most commonly accepted amyloid cascade hypothesis [3] assumes that the initiating pathological event in AD is the deposition of amyloid β peptide (Aβ) leading to the formation of senile plaques (Fig. 1) and the occurrence of tau protein-rich neurofibrillary tangles, resulting in neuronal death and, eventually, dementia. Recently, attention has been drawn to soluble Aβ oligomers (Aβo) [4,9–11], which are considered the neurotoxic form of Aβ. Memory disturbances occurring already at the onset of AD are ascribed to the disruption of synaptic plasticity by Aβo. Later stages of dementia are then a consequence of oligomer-induced degeneration and neuronal death [4]. Other hypotheses of AD differ in the definition of the pathological trigger, which leads to a cascade of events resulting in Aβ plaques and neurofibrillary tangles, the well-known histopathological features of AD. Such trigger could be not only the imbalance in Aβ metabolism, but also cholinergic dysfunction [6], hyperphosphorylated tau protein [5], changes in mitochondrial activity [7] or free radical oxidative damage [8].

Due to the unknown cause of AD and its multifactorial nature, it is difficult to make an early and accurate diagnosis. AD diagnostic criteria were first established by the American National Institute of Neurological and Communicative Disorders (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA) in 1984 [13] and then revised in 2011 [14]. According to these revised criteria, the diagnostics is now based on the assessment of AD biomarkers and a neuropsychological evaluation. Biomarkers are physiological, biochemical or anatomical parameters, which can be measured *in vivo* and reflect the specific features of the pathophysiological processes related to the disease [15]. The ideal biomarker for AD should fulfill the following requirements: It should be focused on the fundamental neuropathology of AD and correlate with histological criteria of the disease. Further, the biomarker is supposed to be based on the presence of the disease itself, not on the risk factor. Importantly, it should allow for an early diagnosis. The method for the detection of the biomarker should be reliable, reproducible, non-invasive and inexpensive. Furthermore, the ideal biomarker should be a base for developing new treatment or help to indicate the efficacy thereof. And it should be able to detect AD with sensitivity of > 80% and distinguish AD from other forms of dementia with specificity of > 80% [16].

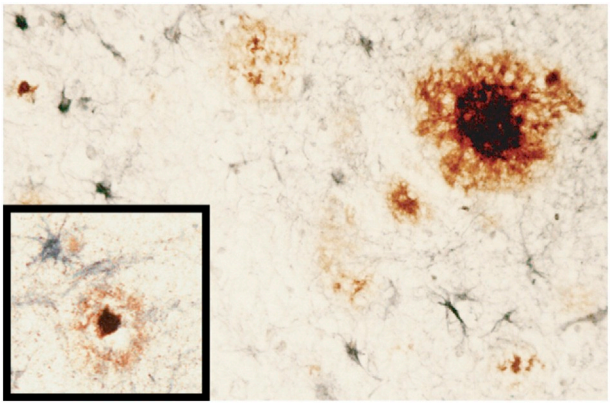

**Fig. 1.** Aβ plaques visualized by immunostaining with antibodies against Aβ in thin tissue paraffin fixed autopsy of AD brain tissue. Reprinted with permission from [12].

## 2. Standard biomarkers of AD

AD biomarkers may be detected either by imaging methods, most commonly magnetic resonance imaging (MRI) and positron emission tomography (PET), or by cerebrospinal fluid (CSF) analysis. The currently established biomarkers of AD can be divided into two groups: biomarkers of Aβ deposition, and neurodegeneration [15]. The first group includes a decreased level of a 42-amino acid form of Aβ ($Aβ_{42}$) in CSF and Aβ deposition in the brain detected via PET using Pittsburgh compound B (PiB). The lower concentration of $Aβ_{42}$ in CSF is ascribed to its aggregation into plaques with less peptide being able to diffuse into CSF [17]. The decrease was found to be approximately 50% compared to a control group [18]. The $Aβ_{42}$ level detected in CSF corresponds with *post mortem* findings of the plaque load [19]. Furthermore, it shows excellent correlation with the results of PiB-PET [20–22]. A limiting factor for the use of Aβ as an AD biomarker could be the fact, that its decrease in CSF was also observed in other types of dementia (dementia with Lewy bodies, Parkinson's disease). It was shown that the $Aβ_{42}/Aβ_{40}$ ratio may better differentiate AD-based dementia from non-AD dementias than CSF $Aβ_{42}$ alone [23]. The other method for the detection of Aβ deposition, amyloid PET imaging, uses $^{11}$C-labeled PiB, which specifically binds to fibrillar Aβ. High retention of this compound in the brains of AD patients leads to an increased signal in the PET scan directly visualizing Aβ plaque accumulation (Fig. 2) [24].

The second group of biomarkers, biomarkers of neurodegeneration, includes an increased level of CSF tau, both total (tTau) and phosphorylated (pTau), decreased fluorodeoxyglucose uptake in PET (FDG-PET) in specific brain areas, and cerebral atrophy detected by MRI [15].

Tau is an intraneuronal protein, which binds to tubulin during its polymerization into microtubules, thereby stabilizing them. Under the pathological conditions of AD, hyperphosphorylation of tau occurs,

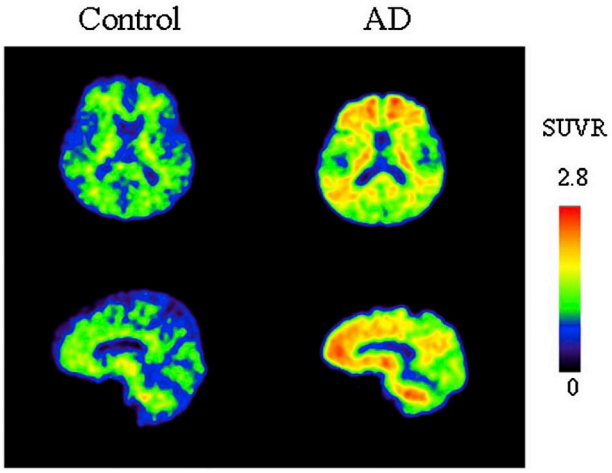


**Fig. 2.** (A) $^{11}$C-PiB standardized uptake value ratio (SUVR) images showing a difference between the $^{11}$C-PiB retention in the brain of a healthy control person (left) and an AD patient (right). Reprinted with permission from [25].

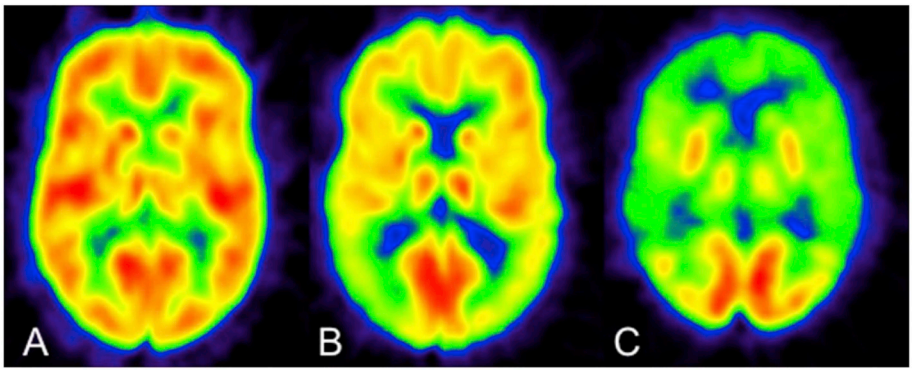


**Fig. 3.** (A) FDG PET of a cognitively normal person; (B) of a patient with suspected early AD; (C) and of a patient with advanced AD. PET scan of the healthy control subject demonstrates expected distribution pattern of cortical FDG uptake. Widespread reduction in regional cerebral metabolic rate of glucose in the parietal and in the frontotemporal and basal ganglia is seen in the suspected AD patients and severe impairment of glucose metabolism occurs in the PET scan of the patient with advanced AD. Reprinted with permission from [38].

leading to the formation of neurofibrillary tangles [26]. Increase in the CSF tTau level is associated with acute brain disorders, such as stroke [27] or traumatic brain injury [28], with the increase corresponding to the severity of the brain tissue damage. Thus, the increased concentration of the CSF tTau is not specific for AD but it is a general indicator of neuronal damage [29]. Nevertheless, the concentration of this protein in CSF correlates with the severity of the disease and high levels are also associated with fast progress from mild cognitive impairment (MCI) to AD and high mortality of such patients [30]. Phosphorylated tau is more specific to AD, as it reflects the phosphorylation state of the tau protein in the central nervous system [29]. The level of CSF pTau corresponds with the amount of neurofibrillary tangles detected *post mortem* [31].

PET using FDG is used to detect the disruption in glucose metabolism indicating neuronal damage and synaptic dysfunction (Fig. 3.) in the brain of AD patients [32]. It was shown [33] that the rate of decrease in FDG uptake corresponds with the level of cognitive impairment along with the progression from normal cognitive state to AD [34]. With the disease progress, the disruption in glucose metabolism in the brain of AD patients is detectable as specific patterns, typically in the posterior cingulate, the parieto-temporal cortices and in the frontal lobes [35]. Reduction in glucose uptake is not AD-specific and occurs also in other types of dementia or neurological disorders [36]. However, vascular dementia, for example, can be distinguished from AD by $^{18}$FDG-PET because the reduction of glucose metabolism occurs within other regions of the brain [37].

Structural MRI is used in AD diagnostics to visualize progressive cerebral atrophy, which is a characteristic feature of AD caused by dendritic and neuronal loss (Fig. 4) [39]. Although MRI is also not specific for AD, the pattern of neuronal damage differs between diseases [39]. Volumetric measurements showed that the severity of neuronal atrophy corresponds with the level of cognitive impairment along the progression from normal cognitive state to AD [34,40]. The first manifestations usually occur in the entorhinal cortex followed by the hippocampus, amygdala, and parahippocampus (regions responsible for memory) [39,41]. Typically, at the time of the diagnosis, atrophy of the abovementioned brain regions is already widespread in most patients [39,42].

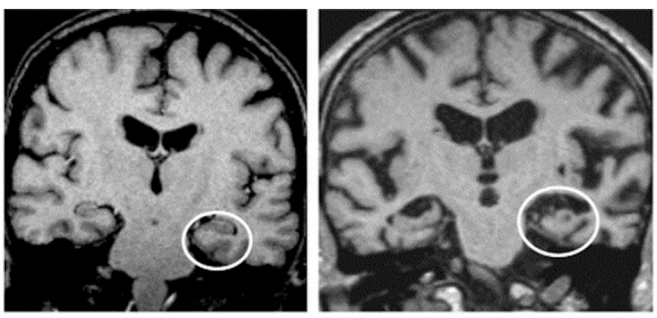

**Fig. 4.** MRI brain scans indicating the absence (left) and presence (right) of medial temporal lobe atrophy. Reprinted with permission from [43].

Several studies [44–47] were performed to assess sensitivity and specificity of CSF biomarkers and their combination. Most of the results vary from 70% to 90% in both sensitivity and specificity [48]. To acquire higher predictive value, the CSF biomarkers and imaging techniques are often combined. Despite high diagnostic accuracy, there are considerable limitations in both CSF analysis and imaging techniques. Brain imaging is expensive, time-consuming and the equipment is not always available, especially for PET. PET is also considered an invasive method, due to the presence of radionuclide in the brain and the use of PiB is limited by its short half-life (~20 minutes) [49].

CSF for biomarker analysis is collected via lumbar puncture, which is also a highly invasive procedure. Thus, the benefits and drawbacks should always be considered. The invasiveness also limits the use of CSF for screening and clinical trials. Furthermore, there is great variability in the results of some studies and the measurements between laboratories [48]. This variability may be caused by the differences in preanalytical sample handling, analytical procedure or differences between immunoassay kits. Unfortunately, such inconsistencies and the lack of standardization make it difficult to interpret the results and to establish a clear limit for the CSF biomarkers [50].

## 3. Novel CSF biomarkers

Despite the invasiveness of the CSF collection, it is still the most accurate source of information about neuropathological changes occurring during neurodegenerative diseases because of a direct contact with the brain. For this reason, searching for novel CSF-based AD biomarkers is still relevant. At this moment, there are several promising candidates, whereas some other molecules have shown to be unsuitable.

Currently, one of the most promising CSF biomarkers is neurofilament light chain (NFL). Neurofilaments are cytoskeletal proteins of neurons and their presence in CSF or blood is a sign of neuronal loss. Although neuronal damage can also occur from other causes, such as inflammatory diseases or brain injury, the NFL levels in CSF and serum were assessed as a potential biomarker of AD. Gaiottino et al. [51] measured the concentration of NFL in serum and CSF of patients with AD, patients with inflammatory diseases, neurological patients with no evidence of structural brain damage and healthy controls. The obtained data showed a correlation between CSF and serum levels of NFL in patients with structural central nervous system (CNS) damage. Serum levels were also significantly increased compared to healthy controls. However, the results were not statistically significant for AD diagnostics and further investigation is needed. Later, Zetterberg et al. [52] reached similar results with a more longitudinal study and proposed the CSF levels of NFL as a marker of AD progression.

Similarly, the presence of neuron-specific enolase (NSE) in CSF is a mark of neuronal damage. This glycolytic enzyme is involved in neuronal metabolism and is physiologically not contained in the

extracellular space [53]. For this reason, Schmidt et al. [53] investigated CSF NSE as a potential AD biomarker. The NSE levels were significantly higher in the AD patients compared to healthy controls, and correlated significantly with tau protein levels. Using the cut-off value of 15.80 $ng \cdot ml^{-1}$, the authors reached 94% sensitivity and 97% specificity. On the other hand, the analysis of the NSE serum levels showed no difference between the AD patients and healthy controls [54].

Another potential CSF biomarker of AD is monocyte chemoattractant protein-1 (MCP-1, also known as chemokine CCL2), which is produced by glial cells and has an important role in inflammatory processes. Increased expression of MCP-1 in patients with AD is most probably in connection with a gradual aggregation of Aβ [55]. Elevated levels of MCP-1 are again significant in CSF, but not in blood serum. Kim et al. [56] measured plasma levels of inflammatory markers in AD patients and age-matched healthy controls observing increased levels of plasma MCP-1 in the patients. However, their results did not reach statistical significance. On the other hand, Galimberti et al. [57] proved MCP-1 to be increased in the serum of patients with MCI and the patients suffering from AD, except for the severe stage of AD, which concurs with the development of Aβ deposition. Another study carried out by Lee et al. [58] showed more promising results with plasma MCP-1 levels gradually increasing with the stage of AD. In addition, the authors found a correlation between MCP-1 levels and the pace of cognitive decline in the patients.

Several studies [59–62] have also focused on β-site amyloid precursor protein cleaving enzyme 1 (BACE1). This enzyme is responsible for the production of Aβ by specifically decomposing the amyloid precursor protein. BACE1 activity was found to be increased in *post mortem* samples of brains of AD patients [59]. Similarly, Zetterberg et al. [60] performed a study comparing BACE1 activity in CSF of AD patients, healthy controls and subjects with MCI. The activity was increased in AD patients and also in subjects with MCI, who progressed to AD later during the follow-up study. On the opposite, in patients with stable MCI and patients who developed other types of dementia, the BACE1 activity was not increased. In addition, results published by Wu et al. [61] showed that the BACE1 activity also correlates with the age of the subjects in both patients and healthy controls. Interestingly, the level of BACE1 activity is not correlated with Aβ levels, but corresponds to tau protein levels in CSF, as shown by Savage et al. [62]. This research group also proved CSF BACE1 activity not efficient enough to become a new diagnostic marker of AD.

Another potential CSF biomarker of AD is glial fibrillary acidic protein (GFAP), one of the proteins of the cytoskeleton of astrocytes. These cells increase the expression of GFAP when activated by the deterioration of the CNS tissue [63]. CSF GFAP levels were found to correlate inversely with the cognitive function of the subjects and an increase in the GFAP production was observed in patients with AD, frontotemporal dementia and dementia with Lewy bodies [64]. Therefore, GFAP might find its use in the prediction of the disease progression [65].

One of the markers proposed to differentiate AD from vascular dementia is the ratio of albumin in CSF and blood serum, which reflects the degree of blood-brain barrier (BBB) dysfunction [66]. An increased CSF/serum albumin ratio was reported in inflammatory and cerebrovascular diseases, vascular dementia and other types of dementia. On the contrary, it remained unchanged in healthy subjects and AD patients [67,68].

## 4. Noninvasive biomarkers

### 4.1. Salivary biomarkers

The composition of human saliva can reveal the health state of a patient and it is already the preferred body fluid in several types of testing, such as hormonal analyses or drug testing [69]. The discovery of salivary biomarkers of AD, which are reliable and robust enough, might bring many advantages, as the collection of saliva is noninvasive, convenient and cost-effective.

As Aβ is a well-established AD biomarker in CSF, its levels in saliva were also evaluated. Bermejo Pareja et al. [70] compared the concentration of salivary $A\beta_{42}$ and $A\beta_{40}$ in AD patients, patients with Parkinson's disease and healthy controls. The $A\beta_{40}$ levels were similar for all participants of the trial, but the levels of $A\beta_{42}$ varied. A decrease was observed particularly in patients in the mild and moderate stage of AD. On the other hand, salivary $A\beta_{42}$ levels in patients with severe AD were close to levels in healthy controls, which corresponds to the changes in the concentration of this marker in CSF as AD progresses [20,71].

Similarly to Aβ, tau protein was also examined as a salivary biomarker of AD. On a cohort of 59 subjects, Min Shi et al. [72] used mass spectrometry to prove an altered concentration of tau protein species in AD patients. Specifically, the pTau/tTau ratio was significantly increased in AD patients compared to healthy controls. In contrast, the concentration of both protein species was elevated in CSF of AD patients [73].

The catalytic activity of acetylcholinesterase (AChE) is significantly lowered in the afflicted brain regions of AD patients, which can be assessed using PET [74]. Therefore, Sayer et al. [75] examined the possibility of measuring AChE activity in human saliva by the Ellman colorimetric method, which could lead to better cost-effectiveness. The results showed a significant decrease in the AChE activity in AD patients compared to age-matched control subjects. However, some patients in this study were on AChE inhibitor therapy. Using the same method, Bakhtiary et al. [76] reached different results. They compared salivary AChE activity of 15 healthy subjects and 15 AD patients on memantine therapy, which does not affect the enzymatic activity. Although the salivary AChE activity was lower in AD patients, the difference was not statistically significant. Similar results were achieved by Boston et al. [77], whose cohort included 15 AD patients, 13 age-matched controls and 13 patients with vascular dementia, only one of which was receiving anticholinergics. The resulting data did not show any statistically significant difference between the groups.

In a recent study by Carro et al. [78], lactoferrin is presented as a salivary biomarker of high potential. Lactoferrin is a peptide known for its broad antimicrobial effects and the modulation of immune response and inflammation. Lowered levels of lactoferrin in AD patients may be consistent with systemic infection being a known risk factor of AD. Lactoferrin levels in saliva were assessed using enzyme-linked immunosorbent assay (ELISA) resulting in 100% sensitivity and specificity. Moreover, lactoferrin levels were not reduced in healthy controls and patients suffering from Parkinson's disease. In addition, 14 out of 18 healthy participants with levels of lactoferrin close to the cut-off value have developed MCI during the course of the study, thus, potentially making lactoferrin a key biomarker for the early diagnosis of AD.

Although the aforementioned results seem promising, methods of the use of the salivary biomarkers require normalization. As in any other biofluid, the contents may vary depending on a broad variety of factors. In the case of saliva, circadian rhythm, flow rate or the time of sample collection seem to be the most influential. In addition, due to the primary functions of the saliva, including enzymatic degradation, the level of biomarkers is unstable, thus, the samples need to be processed rapidly [69].

### 4.2. Urinary biomarkers

The signs of AD pathology may also be found in the urine. The most prominent changes are caused by oxidative stress connected with the disease and changes in the metabolism of proteins and lipids. In contrast with salivary biomarkers, the use of urine in diagnostics can be easily normalized by relating the concentration of the biomarker to the

concentration of creatinine, which is physiologically stable, thus, accounting for the oscillation in urine flow rate [79]. According to Hartmann et al. [80] the best performance for noninvasive AD detection may be mediated by 8,12-iso-iPF$_{2\alpha}$-VI, total free amino acids, glycine and 8-hydroxy-2'-deoxyguanosine, all of which reached over 90% accuracy.

Isoprostane 8,12-iso-iPF$_{2\alpha}$-VI levels in urine, as well as in plasma and CSF, were studied by Praticó et al. [81] in subjects with AD, MCI and healthy age-matched controls. Isoprostane 8,12-iso-iPF$_{2\alpha}$-VI is generated from fatty acids of grey brain matter by lipid peroxidation by free radicals [82]. All biofluids of patients with AD showed the highest levels of 8,12-iso-iPF$_{2\alpha}$-VI, followed by patients with MCI. Furthermore, five patients with significantly increased levels of 8,12-iso-iPF$_{2\alpha}$-VI converted to AD during the follow-up phase of the study. Therefore, the authors propose to use this biomarker for identifying patients with MCI at risk of rapid conversion to AD. Similar results were achieved by García-Blanco et al. [82].

According to Fonteh et al. [83], urine of patients with AD shows significantly increased levels of free amino acids, especially glycine. Glycine acts as an inhibitory neurotransmitter in the spinal cord and the lower brain stem [83], and its excretion to urine can also be a sign of general deterioration of patient's health condition [84].

Another promising marker for early AD diagnostics seems to be 8-hydroxy-2'-deoxyguanosine (8-OHdG). During oxidative DNA damage, which is one of the pathological processes responsible for the development of AD, 8-OHdG is generated by the oxidation of guanine bases in DNA by the most reactive oxygen species, the hydroxyl radical. Zengi et al. [85] measured 8-OHdG levels in the urine of AD patients and healthy controls by HPLC. Both the 8-OHdG concentration and the 8-OHdG concentration normalized to creatinine level were significantly higher in AD patients, which is in agreement with previous findings [86,87].

## 5. Blood-based biomarkers

Potential biomarkers of AD present in blood have also been studied extensively. These biomarkers represent a cost- and time-effective way to extend or replace the current laboratory tests based on the invasive CSF analysis. An immense advantage of blood biomarkers is that, compared to the lumbar puncture, blood collection is a much less invasive procedure. Therefore, it allows repeated sampling and measurements to monitor the disease progression over the years, or to evaluate the efficacy of the newly developed drugs during clinical trials [88,89]. On the other hand, the BBB represents a major issue in finding applicable blood-based biomarkers. This highly selective semipermeable membrane protects the brain from an intrusion of harmful products and pathogens, but it also restricts the egression of molecules from the brain. Despite that, some small molecules from the brain may be detected in plasma in low concentrations as the exchange between blood and CSF occurs on some level [88]. Furthermore, it has been reported that BBB breakdown occurs in AD [90,91], which leads to its increased permeability.

The most commonly studied plasma biomarker of AD is Aβ. However, the results of several studies [92–94] are inconsistent, making it unclear to what extent Aβ content in plasma corresponds to its concentration in CSF and the presence or stage of AD. Among other things, the low reproducibility of the results is possibly caused by small sample size and its heterogeneity, variability and low sensitivity of the used ELISA [95]. In a recent study [95], an ultrasensitive digital ELISA was used to measure plasma Aβ$_{42}$, Aβ$_{40}$ and their ratio. A decrease in the levels of Aβ$_{42}$ and Aβ$_{40}$ was found in the plasma of AD patients and a decreased Aβ$_{42}$/Aβ$_{40}$ ratio was even noticed in the plasma of patients with preclinical AD. However, neither of these potential biomarkers provided sufficient accuracy to identify AD patients. In another study [96], a new approach for plasma Aβ analysis was tested. Immunoprecipitation coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to measure Aβ$_{42}$/Aβ$_{40}$ and it showed great ability to predict the presence of Aβ plaques in the brains of AD patients (determined by Aβ PET imaging). The correlation between the Aβ$_{42}$/Aβ$_{40}$ ratio and the levels of Aβ$_{42}$ in CSF was also observed. To evaluate the diagnostic relevance of plasma Aβ, a large study including 1040 subjects was realized by Hanon et al. [97]. A decreasing concentration of both Aβ$_{42}$ and Aβ$_{40}$ was observed within different stages of neurodegeneration ranging from non-amnestic MCI to amnestic MCI to AD. Interestingly, the levels of Aβ$_{42}$ in plasma correlated with all established CSF biomarkers only in the case of MCI. In AD, there was a correlation only between plasma and CSF Aβ$_{42}$. It is evident that further research is required to evaluate the diagnostic potential of plasma Aβ.

Several studies [98–100] have also focused on Aβo. Xia et al. [99], for example, developed a specific ELISA, which was able to simultaneously detect Aβo and Aβ$_{42}$ monomers in plasma or in *post mortem* brain tissue. The results showed higher levels of Aβo in samples of AD patients, tight association between the levels of both Aβ forms and, further, their decrease in plasma over 2 years indicating a direct connection between plasma Aβ levels and the formation of amyloid plaques. Another research group [98] found an increased concentration of oligomeric Aβ in the plasma of patients with AD using a newly developed Multimer Detection System. This ELISA enabling the differentiation between multimers and their monomeric forms was also used to measure dynamic changes of Aβo levels in plasma induced by spiking with Aβ$_{42}$ [100]. Synthetic Aβ$_{42}$ was added to the plasma samples of AD patients and normal control subjects and the formation of Aβo was observed over time. The hypothesis derived from the observations was that oligomerization of synthetic Aβ is influenced by different plasma composition of patients with AD and healthy control individuals. This approach enabled the differentiation between the studied groups with sensitivity and specificity of 83.3% and 90.0%, respectively.

Levels of tau protein in plasma were also evaluated. To detect the low concentrations thereof, an ultrasensitive immunoassay technique had to be developed first [101]. A large study [102] showed elevated tau in the plasma of AD patients, which unfortunately overlapped with normal control individuals. In addition, the correlation with increased tau as well as decreased Aβ in CSF was weak. On the other hand, Tatebe et al. [103] reported pTau as a promising biomarker due to its concentrations in plasma being significantly higher in AD patients than in controls. Moreover, there was a strong correlation between plasma and CSF pTau.

Similarly to CSF, blood plasma is a source of a broad range of other molecules potentially involved in AD. Several studies [104–106] focused on the brain-derived neurotrophic factor (BDNF), a protein important for normal functioning of the brain, which is assumed to be involved in the etiopathogenesis of AD [104]. However, the outcomes were contradictory. For example, Levada et. al [105] detected lower levels of BDNF in subjects with AD, while Faria et al. [106] reported higher concentrations. Platenik et al. [107] detected a significantly lower BDNF concentration in platelet-rich plasma, but not in plasma; in AD patients with moderate to severe stages of dementia, but not in AD patients with a mild stage of dementia. This result supported findings that changes in BDNF serum concentrations depend on the severity of AD.

Inconsistent results were achieved in studies of the protein clusterin, which is a molecular chaperone regulating Aβ clearance and aggregation into plaques. Furthermore, it participates in lipid transport during cell differentiation, cell death and modulates inflammation [108,109]. Jongbloed et al. [110] showed that a higher level of clusterin in the plasma of individuals with MCI represents an increased risk of progression to AD. On the other hand, the authors proposed that higher levels of clusterin in AD patients are related to slower cognitive decline. In another study [111], clusterin plasma levels were found to be significantly higher in both MCI and AD patients compared to healthy controls. Weinstein et al. [109] showed a strong association of clusterin

concentration with age. This suggests a higher risk of dementia in older individuals, whereas it has a protective role in younger brains. Recently, Islam et al. [108] developed a label-free immunosensor for the detection of clusterin in plasma with high sensitivity, selectivity and a detection limit far below the average concentrations of this glycoprotein in human plasma.

Equally to Aβ, NFL may be detected in the blood stream, thus, emerging as a promising AD biomarker [112,113]. Axonal damage occurring during AD leads to the release of neurofilaments into the extracellular space and, consequently, into CSF and blood. Lewczuk et al. [112] detected elevated NFL within an MCI group and even higher concentrations thereof in a group of AD patients. Similar results were reported by Mattsson et al. [114]. Furthermore, levels of NFL in plasma correlate with the cognitive state, neuroimaging findings and levels of established biomarkers in CSF [114]. However, elevated NFL is not specific for AD as it was also observed in other neurodegenerative diseases, such as frontotemporal dementia. Therefore, NFL might better serve as a screening tool to rule out neurodegeneration or to monitor the progression of dementia [115]. Among other studied molecules, it is worth to mention soluble neuregulin-1 (sNRG-1) [116] or tumor necrosis factor receptor 1 (TNFR-1) [117], the latter of which functions as a mediator of inflammation and was identified as a potential biomarker of AD from 25 different inflammatory biomarkers.

### 5.1. Proteomics and metabolomics in AD diagnostics

In the last decade, another approach has started to attract attention, aiming on a broad spectrum of analytes at one time instead of a single molecule. In 2007, one of the first studies using proteomic profiling was published by Ray et al. [118], who identified 18 plasma proteins allowing for the differentiation of AD patients from healthy individuals with high accuracy. More recently, O'Bryant et al. developed [119] and later validated [120] an algorithm, which separates AD patients from controls using 21 proteins, including fatty acid binding protein, pancreatic polypeptide, thrombopoietin, tumor necrosis factor-α and several interleukins. In 2016, this algorithm was applied on 1329 samples of multiple community- and clinic-based cohorts [121]. A reference set was created from 1128 samples, which together with demographic information (age, gender, education) allowed for the detection of AD within the validation cohort (201 samples) with a positive predictive value (PPV) and negative predictive value (NPV) of 0.81 and 0.95, respectively. Furthermore, using the 21 proteins, the MCI patients were detected with a PPV of 0.74 and an NPV of 0.93. These results support the usefulness of proteomic profiling, making such blood screen the first step in a diagnostic process to rule out individuals who do not require a more costly and invasive examination [121].

A similar approach, only working with smaller molecules, is metabolomics. It allows for studying a biological system as a whole and monitoring disease-associated molecular alternations, which maximizes the possibility of finding a suitable biomarker [122,123]. Metabolomic analyses are usually performed either by mass spectrometry (MS) coupled with liquid chromatography (LC) or by nuclear magnetic resonance (NMR). Using ultraperformance LC-MS, a panel of 6 plasma metabolites (arachidonic acid; N,N-dimethylglycine; thymine; glutamine; glutamic acid; cytidine) was derived by Wang et al. [124] to differentiate AD patients from healthy individuals. Another five metabolites (thymine; arachidonic acid; 2-aminoadipic acid; N,N-dimethylglycine; 5,8-tetradecadienoic acid) were used to identify MCI. Graham et al. [125] provided a metabolic analysis of 72 plasma samples and, subsequently, created a statistical model capable of accurately identifying MCI in patients, who would later convert to AD. Further, they observed specific changes in L-arginine and polyamine metabolism related to progression to AD. Toledo et al. [126] analyzed more than 700 samples of blood serum using ultraperformance LC coupled with tandem MS. In their study, several metabolites altered in AD were identified, including acylcarnitines and some amines. Levels of sphingomyelins and ether-containing phosphatidylcholines varied already in the preclinical stage of AD. A five-year longitudinal study [127] identified 24 metabolites, including 22 lipids, asparagine and asymmetric dimethylarginine, which allowed for the prediction of conversion from normal cognitive state to MCI or AD with high reliability (the area under the curve (AUC) of the receiver operating characteristics reached 0.995 after internal validation).

The NMR-based metabolomics was applied on plasma samples of mouse models several times [128–130]. Graham et al. [128] observed significantly altered levels of acetate, citrate, glutamate, glutamine and methionine in the plasma of mice from the transgenic mouse model. According to Kim et al. [129], several metabolites, including pyruvate and creatine, were reduced in AD model mice compared to controls. Further, in the large NMR metabolomic study [131] of human plasma samples, including over 20 000 participants, 228 metabolites, lipids or lipoproteins were quantified and several metabolites associated with either lower (isoleucine, leucine, valine, creatinine, one subclass of very low-density lipoproteins (VLDL)) or higher (one subclass of each VLDL and high-density lipoproteins (HDL)) dementia risk were identified. In another study [132], 15 metabolites, including a subclass of HDL, docosahexaenoic acid, ornithine, glutamine and glycoprotein acetyls, were reported to increase the risk of dementia. In addition, the NMR-based metabolomic profiling of samples of CSF [133] and saliva [134,135] has also demonstrated great potential for the discrimination between AD patients and non-demented individuals.

### 5.2. Vibrational spectroscopy

Vibrational spectroscopic techniques are fast, reliable, cost-effective, non-destructive and they require only a small amount of sample with minimal sample preparation, which are ideal characteristics for the use in clinical practice. Similarly to proteomic and metabolomic approach, spectroscopic techniques allow a simultaneous study of all molecules present in the sample, instead of examining individual molecules separately. Therefore, they are ideal for the diagnostics of complex, multifactorial diseases, such as AD [89]. Furthermore, the spectra contain information about not only the chemical composition of the analyzed sample, but also the molecular structure [136]. The differences between the measured spectra of plasma of AD patients and healthy individuals are usually minute. Therefore, it is crucial to pay special attention to the quality and reproducibility of the spectra [137]. The discrimination of the samples based on the spectral differences is typically accomplished by multivariate statistical methods, such as linear discriminant analysis (LDA) or principal component analysis (PCA).

The most frequently used molecular spectroscopic technique in AD research is probably infrared (IR) spectroscopy. There are two major bands in the typical IR spectrum of blood plasma (Fig. 5), both of which are sensitive to the secondary structure of proteins. The first band, usually occurring in the region of 1670–1620 $cm^{-1}$, is called amide I and is associated with the C=O stretching of the peptide bond. The second, amide II, with the maximum at ~1545 $cm^{-1}$, arises from the combination of N-H bending and C-N stretching. Furthermore, a band at ~1455 $cm^{-1}$ can be assigned to the bending of $CH_2$ and $CH_3$ groups of the phospholipid and protein side chains. Several bands may be found in the range of 1260–1400 $cm^{-1}$, which is related mainly to the symmetric stretching of the $COO^-$ group and the symmetric bending of the methyl group in proteins. The band with the maximum at ~1245 $cm^{-1}$ then includes the $PO_2^-$ asymmetric stretching of the phospholipid group [137,138].

Nabers et al. [136] tested the use of an immunosensor with an IR detection for the study of CSF and blood plasma of patients with AD using attenuated total reflection (ATR) Fourier transform infrared (FTIR) spectroscopy. Antibodies that selectively bind Aβ were applied to the surface of the ATR crystal in the flow cell. In the previous study [139] using the same infrared sensor, Nabers et al. showed that the

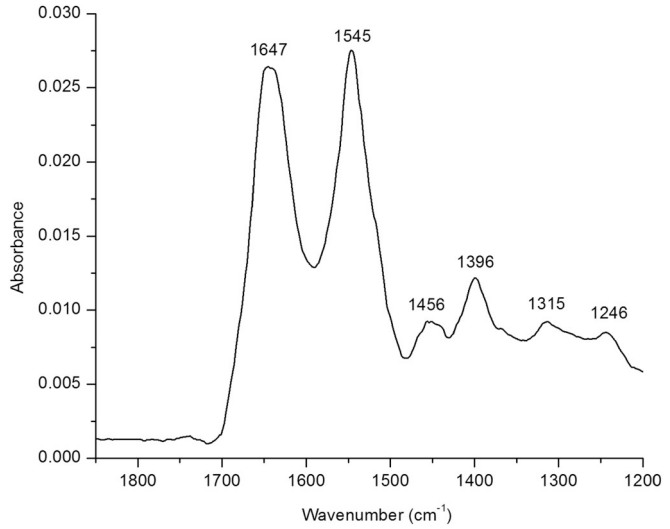


**Fig. 5.** Typical IR spectrum of the blood plasma of a healthy individual in spectral region 1800–1200 $cm^{-1}$.

secondary structure of Aβ can be distinguished. The synthetic monomeric Aβ with α-helical structure was characterized by an amide I band at 1655 $cm^{-1}$, whereas amyloid fibrils showed a downshift to 1628 $cm^{-1}$ with a second band at 1664 $cm^{-1}$ indicating β-sheet structure (Fig. 6). As the misfolding and aggregation of Aβ into insoluble fibrils is associated with AD, the amide I band was used to distinguish patients with AD from healthy individuals. In the spectra of blood plasma of AD patients, a significant shift of the amide I band to lower wavenumbers was observed. The threshold for classification was determined at 1643 $cm^{-1}$, where every subject with an amide I band maximum below this value was classified as AD patient. This approach led to the identification of the samples with 85% accuracy, 75% sensitivity and 88% specificity after cross-validation. Furthermore, 90% accuracy, 88% sensitivity and 93% specificity were determined for CSF analysis after validation on another sample set. In addition, an increase in levels of Aβ in the β-sheet conformation was observed in 11 samples of patients diagnosed with MCI due to AD. Therefore, this method might allow for AD diagnostics already at this initial stage of the disease.

In another study [137], FTIR spectroscopy was used to analyze not only blood plasma, but also isolated white blood cells (WBCs). The observed spectral differences between healthy controls and AD patients were more noticeable in the spectra of blood plasma than in those of WBCs. In the plasma spectra, the most significant changes occurred in the symmetric stretches of $PO_2^-$ (1084 $cm^{-1}$), $COO^-$ in amino acids, and in the $CH_3$ scissoring of the protein methyl group (1395 $cm^{-1}$). On the contrary, the WBCs spectra showed the largest differences in the $PO_2^-$ symmetric (1084 $cm^{-1}$) and antisymmetric (1236 $cm^{-1}$) stretches. For the classification of the samples, PCA followed by LDA was used. This approach allowed for the identification of the AD patients with 72% sensitivity, 81% specificity and 77% accuracy when using the plasma spectra, and with 82% sensitivity, 88% specificity and 85% accuracy for the WBC spectra (values after cross-validation). Interestingly, despite more pronounced spectral differences in the plasma, better classification results were obtained for the WBC measurements. Subsequently, the classification was also performed after the mild or both mild and moderate stages of AD were excluded. The obtained values were higher, indicating that the largest differences exist between healthy subjects and patients with severe AD.

FTIR spectroscopy was used to analyze blood plasma also by Paraskevaidi et al. [89]. The ATR technique was applied, and various multidimensional statistical methods were tested. The goal of this study was not only to distinguish AD patients from healthy controls, but also to separate AD from other types of dementia, such as dementia with Lewy bodies or frontotemporal dementia. The study included 347 patients with different types of dementia and 202 age-matched healthy control subjects. The AD patients were identified with specificity and sensitivity of 70%. After the information about apolipoprotein E genotype was included, the value increased to 86%. AD with early onset (patients under 65 years of age) was recognized with 80% sensitivity and 74% specificity, AD separation from dementia with Lewy bodies was possible with 90% sensitivity and specificity (after cross-validation). The best discrimination of samples was achieved by a genetic algorithm followed by LDA for the fingerprint region and PCA followed by quadratic discriminant analysis (QDA) for the higher wavenumber

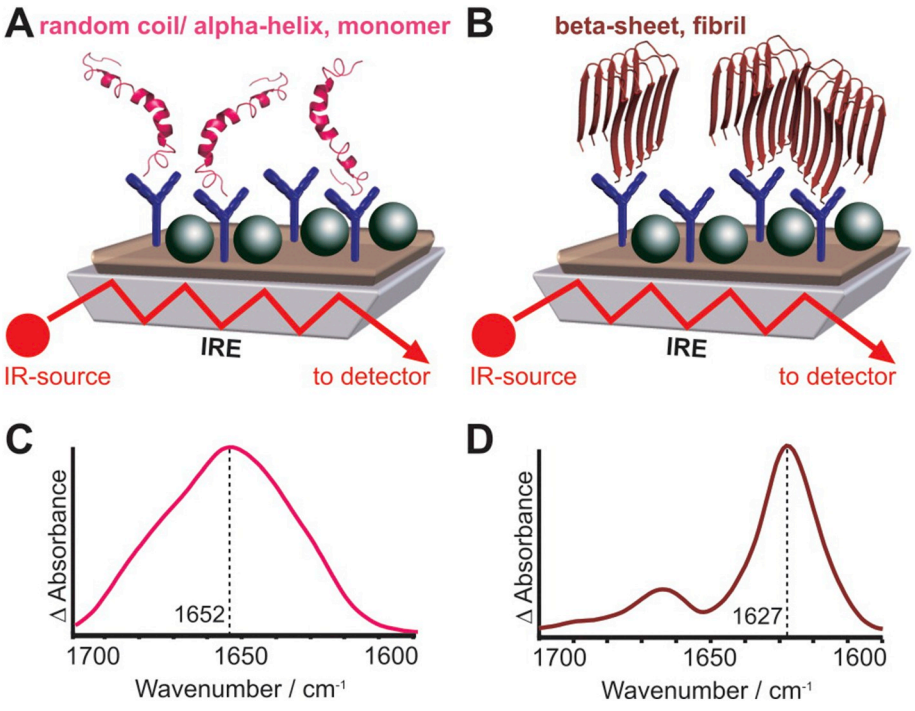


**Fig. 6.** Schema of the immuno-ATR-FTIR spectroscopic setup. Tethered antibodies capture Aß peptides out of liquid samples for the detection of the infrared absorbance (C, D) of monomerized (A, C) and fibrillized (B, D) synthetic Aß. The shift of the amide I band to lower wavenumbers is induced by the ß-sheet structure of the fibrillized sample (D). Reprinted with permission from [136].

region.

Near infrared (NIR) spectroscopy was used to analyze plasma of patients with AD by Burns et al. [140]. Five spectral bands sensitive to oxidative changes were selected to create a regression model for the NIR spectra evaluation. These selected spectral bands were associated with the presence of the functional groups of heme, R-CH, R-OH, R-NH and $H_2O$. In total, 63 plasma samples from patients with AD, individuals with MCI and control subjects were examined. The regression model enabled distinguishing of the AD patients from healthy controls with sensitivity and specificity of 80% and 77%, respectively. A group of MCI patients was not separated in the statistical model and these patients were almost evenly classified into the other two categories. Further, it was observed that the measured spectra were not influenced by gender, age nor sample storage time.

Other study using NIR spectroscopy was realized by Paraskevaidi et al. [141]. Blood plasma (284 samples) was analyzed with the aim to provide further evidence about the diagnostic potential of the spectroscopic methods. The acquired spectroscopic data were processed by PCA-QDA. The most significant spectral differences were found mainly in the regions related to proteins (~1860 nm – combination of OH stretch and C-O stretch second overtone; ~1908 nm – C═O stretch second overtone in peptides; 2100, 2111 and 2150 nm – combination of bands consisting of N—H bend second overtone, CH stretching and C═O stretching, and combination of C═O stretching, N-H in-plane bending and C—N stretching in proteins). The created statistical model allowed for the identification of the AD patients and healthy controls with 93% accuracy, 88% sensitivity and 96% specificity after validation, additionally the AUC value reached 0.928. Furthermore, this study showed the possibility to use inexpensive low-E glass slides as a substrate and then subtract its signal from the sample spectra without affecting the diagnostic result.

Raman spectroscopy represents another vibrational spectroscopic technique widely tested in the context with AD diagnostics. A typical Raman spectrum of blood plasma (Fig. 7), measured using visible excitation (532 nm), contains three intense bands with maxima at 1519, 1158 and 1007 $cm^{-1}$. These bands can be assigned to C═C and C—C stretching vibrations of carotenoids [142]. These are found in blood plasma only at low concentrations but due to visible excitation, resonance enhancement occurs, and the bands are intense in the Raman spectrum [143,144]. Important regions sensitive to protein secondary structure include the amide I band at ~1658 $cm^{-1}$ corresponding to the stretching of the C═O group; the extended amide III region (1340–1230 $cm^{-1}$) attributed to the in-phase combination of in-plane

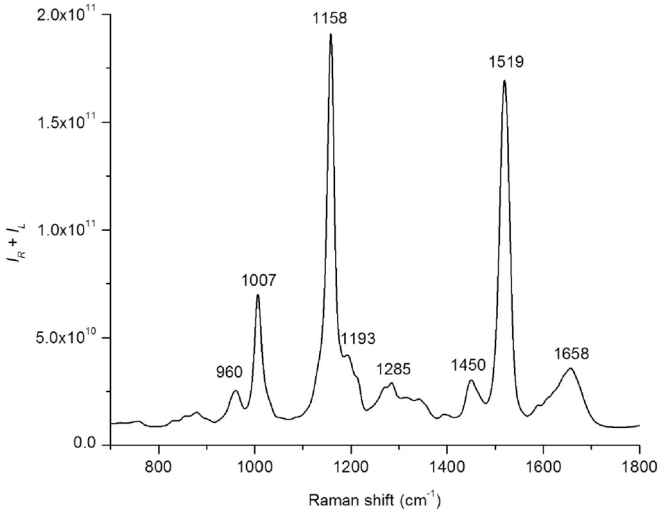


**Fig. 7.** Typical Raman spectrum of blood plasma of a healthy person in the spectral region of 800–1800 $cm^{-1}$ measured at an excitation wavelength of 532 nm.

N—H and C—H bending with C—N stretching, and the C—C stretching region represented by a band at ~960 $cm^{-1}$. The band with a maximum at ~1450 $cm^{-1}$ may be assigned to the stretching of the $CH_3$ and $CH_2$ groups of the aliphatic side chains of amino acids and phospholipids. The spectrum also includes less intense bands typical for aromatic amino acids (1618–1605, 1206, 1193, 1004, 880–850 $cm^{-1}$) [142].

The information obtained from Raman spectra is, to a certain extent, complementary to that provided by the IR spectra. The great advantage of Raman spectroscopy, compared with IR spectroscopy, is the possibility of measurements in an aqueous environment and also the possibility of using glass cells and other optical elements. On the other hand, the high energy of the incident photons can cause electron excitation of the molecule and subsequent fluorescence, which leads to an increase of the background in Raman spectra or even completely overlaps them. This issue may be solved by using radiation of lower energy (NIR), an addition of quenching agents or irradiating of the sample by high-power laser for a prolonged time [145]. Tatarkovič et al. [146] developed a new method for reducing the fluorescence during blood plasma measurements. The combination of sodium iodide as a fluorescence quencher and photobleaching led to a reduction of the spectral background by 90%.

Carmona et al. [138] observed significant differences between both Raman and IR spectra of blood plasma of 50 AD patients and 14 control subjects. In both types of spectra, changes in the amide I band were noticeable. The maximum at ~1658 $cm^{-1}$ is assigned to the α-helical structure, which can be attributed to human serum albumin, the most abundant plasma protein with 67% α-helical structure. In both Raman and IR spectra of plasma of AD patients, this band was influenced by elevated levels of β-sheet proteins, which the authors attribute to the formation of the Aβ peptide and to the increasing ratio of β-sheet-rich globulins ($\alpha_1$-antitrypsin, transferrin, γ-globulins) and albumin. Moreover, in the IR spectra, changes were also observed within 1200–1000 $cm^{-1}$, which corresponds to C—O stretching vibrations of hydroxyl compounds (isoprostanes, hydroperoxides) that are suggested to be the products of lipid peroxidation caused by oxidative stress during AD. In the Raman spectra, another band showing sensitivity to AD arose near 938 $cm^{-1}$, which is also associated with the secondary structure of proteins. LDA was applied on the spectroscopic data distinguishing AD patients from healthy individuals with a diagnostic accuracy of 94% (after validation of the statistical model).

Another study [147] evaluated the potential of Raman spectroscopy of blood plasma for the discrimination of AD and dementia with Lewy bodies from controls as well as from each other. These two diseases have some similar symptoms and clinical characteristics; therefore, they can be easily misdiagnosed. A total of 56 samples divided into four groups (early and severe stage of AD, dementia with Lewy bodies, healthy controls) were analyzed and spectral data were subsequently processed using cross-validated PCA-LDA and a support vector machine algorithm. Six different statistical models were created to compare all groups. Both sensitivity and specificity ranged between 80% and 90% in most of the cases. Further, spectral bands that were significant for the discrimination were identified, e.g. amide I (~1650 $cm^{-1}$) or amide II (~1530 $cm^{-1}$), the latter of which was up-shifted in the spectra of both the early and the severe stage of AD. The authors attribute this observation to an increase in tau protein or NFL in plasma. In addition, lower intensity of the band at ~1432 $cm^{-1}$ was observed, which was suggested due to the decrease of lipid levels caused by the oxidative stress-induced damage of phospholipid membranes. Further, the level of phenylalanine increased in dementia with Lewy bodies compared to healthy controls.

Other non-AD neurodegenerative diseases were also included in a study by Ryzhikova et al. [148] analyzing blood serum. An artificial neural network was used to classify the spectroscopic data, the learning of which, as well as the subsequent validation, took place on subsets of the measured data. The neural network was able to distinguish between AD, other forms of dementia and healthy controls with specificity and

sensitivity of more than 95%. However, the neural network algorithm was unable to determine the regions in the Raman spectra, which would be of importance for this differentiation. Thus, this method has limited significance for interpreting the spectra from a biochemical point of view. Therefore, a genetic algorithm was used, allowing to find specific spectral regions that were most significant for the discrimination of the measured spectra.

Raman and IR spectroscopy have not been used only for plasma samples, but the CSF of AD patients was also analyzed [149]. Moreover, Aβ and other hallmarks of AD were studied. For example, Goldblatt et al. [150] observed conformational changes of Aβ during its aggregation using FTIR spectroscopy; and Raman spectroscopy allowed for the detection of structural changes of Aβ in rat hippocampus caused by oxidative stress [151] or imaging of senile plaques and neurofibrillary tangles in brain tissue [152].

### 5.3. Chiroptical spectroscopy

Chiroptical methods are based on the interaction of chiral molecules with linearly or circularly polarized radiation. The most significant are circular dichroism (CD) and Raman optical activity (ROA).

The principle of CD spectroscopy is a different absorption of the left- and right-handed circularly polarized radiation by a chiral molecule. When UV or visible radiation is used, electron transitions occur, which are characteristic for electronic circular dichroism (ECD). Similarly, infrared radiation triggers vibrational transitions and vibrational circular dichroism (VCD) is measured. Using ECD, only chiral molecules that contain a chromophore can be examined, while the VCD signal is provided by all optically active molecules [153,154]. ROA is based on the measurement of the difference between the intensity of right- and left-handed circularly polarized radiation in the spectrum of non-elastically scattered radiation. The ROA experiment can be arranged in a variety of ways. In the so far only commercially available device, the intensity of right- and left-handed circularly polarized components in backscattered light is measured using unpolarized incident radiation [155]. In ROA, the transitions occur between vibrational levels, so the ROA spectrum is provided by all optically active molecules. In both VCD and ROA, a molecule undergoes vibrational transition, therefore these spectroscopic techniques are collectively referred to as vibrational optical activity (VOA) [156]. One of the advantages of ECD and ROA is the ability to perform the analysis in an aqueous environment, making it easy to achieve in vivo conditions, which is important for understanding the biological functions of molecules. On the contrary, the experiment is demanding and a costly device is required especially for ROA. In addition, both Raman spectroscopy and ROA struggle with undesirable fluorescence [155,156].

Chiroptical methods are sensitive to the spatial structure of chiral molecules. Therefore, these methods have high potential for the study and diagnostics of AD, such as monitoring the accumulation of the Aβ protein in abnormal conformation as a typical feature of AD. The profile of the ECD spectrum of proteins is dependent on their secondary structure. For α-helix, a typical positive band arises at 193 nm and two negative, partially overlapping bands are observed at 222 and 208 nm, while the proteins with the predominant β-sheet structure have a negative band at 218 nm and a positive band at 195 nm [157]. In VCD, vibrations of the peptide bond are the most significant for determining the secondary structure of proteins, i.e. amide I (1670–1620 $cm^{-1}$) and amide II (1600–1500 $cm^{-1}$) [156]. The amide I band is also present in the ROA spectrum, in which the next significant region of amide III may be detected in the interval between 1300–1230 $cm^{-1}$ corresponding to a combination of C—N stretching and N—H in-plane bending [155].

Chiroptical methods (ECD, ROA) supplemented with infrared and Raman spectroscopy were uniquely used to analyze samples of plasma of AD patients and control subjects by our group [158]. Differences between AD and control samples were observed in all types of measured spectra (ROA spectrum in Fig. 8). Acquired spectral data were subsequently evaluated using LDA, which resulted in the differentiation of AD patients from non-demented control subject with sensitivity and specificity of 94% and 84%, respectively (after leave-one-out cross-validation).

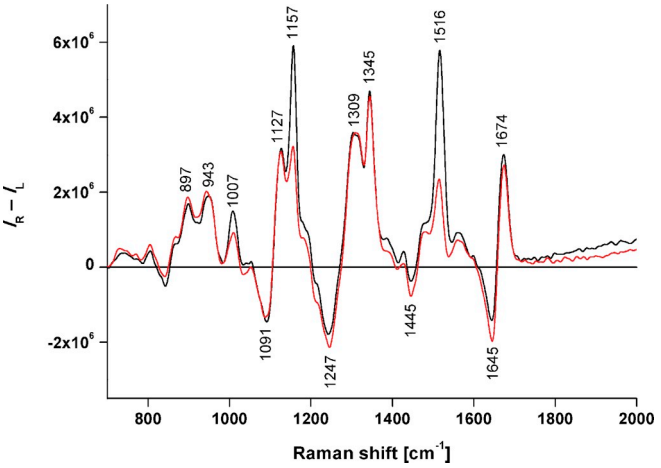


**Fig. 8.** Comparison of average ROA spectra of blood plasma of AD patients (red) and healthy controls (black). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

## 6. Conclusion

Despite the high diagnostic accuracy of the currently established biomarkers of AD, there is still need for one, which would allow for a minimally invasive, cost- and time-effective early detection of this neurodegenerative disease. Moreover, the biomarker should be specific for AD. Several promising molecules present in CSF, blood or other biofluids, such as saliva and urine, have been identified in the past years. The highest potential was shown for NFL in both CSF and blood plasma, lactoferrin in saliva or Aβ, pTau and Aβo in blood plasma. Encouraging results have also been achieved in studies using proteomics or metabolomics.

Considerable attention has been paid to molecular spectroscopy of blood plasma, which had demonstrated its high efficacy in AD diagnostics. Spectroscopic techniques allow the study of numerous molecules present in the sample at the same time and, furthermore, they provide simple, rapid, low-cost measurements with minimal need for sample preparation. When applied to biofluids collected with minimum invasiveness, such as blood plasma, spectroscopic methods may serve as a tool for screening of groups at risk of AD or for the monitoring of the disease progression.

Nevertheless, there are several issues related to most of the presented potential AD biomarkers. Often, these biomarkers originate from a single study or there is significant inconsistency in the results from different studies. Moreover, most of the studies include an insufficient number of probands. Thus, if any of these novel biomarkers should become part of the clinical practice, there is a need for large-scale cross-laboratory studies. In addition, the pre-analytical sample processing should be standardized, analytical methods validated and the impact of other factors on the biomarker levels, such as age, ethnicity, comorbidities and concomitant medications, must be thoroughly evaluated.

## Conflict of interest

The authors have no conflict of interest to declare.

## Acknowledgement

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