Publishable Summary for 15HLT02 ReMiND
Role of metals and metal containing biomolecules in neurodegenerative diseases such as Alzheimer's disease

Overview

Due to an ageing population, neurodegenerative diseases such as Alzheimer's disease (AD) are a major challenge facing the health care system, currently affecting over 6 million people in the European Union. Diagnostic methods used for the identification and quantification of relevant AD biomarkers require improved accuracy in order to help treatment in early stages of the disease. This project focussed on the development of potential reference measurement procedures for these biomarkers based on isotope dilution to improve the reliability and comparability of the results in clinical laboratories.

Need

Due to an ageing population, neurodegenerative diseases are one of the major challenges facing the health care system, currently affecting over 6 million people in the European Union and 44.4 million people worldwide.

The most common cause of dementia is Alzheimer's disease (AD), representing 70 % of all cases. Studies have shown that treatment for the disease is most promising in the early stages, However, due to a lack of accuracy in diagnostic methods used for identification and quantification of relevant biomarkers only half of the patients suffering from AD are currently identified, and they are often in the advanced stages of the disease.

The most established biomarkers for AD are β-amyloid peptide 1-42 (A β1-42), β-amyloid peptide 1-40 (A β1-40), total tau-protein (T-tau), hyperphosphorylated tau-protein (P-tau), and ratios thereof, measured in cerebrospinal fluid (CSF). As these markers are also formed to some extent during normal ageing, cut-off values are needed by clinicians to distinguish between healthy and diseased individuals. These biomarkers are commonly determined using immunoassays or optical methods but often give inconsistent results and high variabilities, of up to 25 % for one specific method between laboratories prevents these universal cut-off values being established.

Objectives

The aim of this project was to provide reference measurement procedures for the identification and quantification of relevant biomarkers for the diagnosis of neurodegenerative diseases such as AD.

This project addressed the following scientific and technical objectives:

1. **To develop methods for the traceable quantification of metals and metal containing biomolecules** of neurodegenerative diseases at µg/L levels or below and in small µL sample volumes.
2. **To produce and characterise isotopically labelled spike materials** for metals and metal containing biomolecules of neurodegenerative diseases.
3. **To develop new and accurate methods for measuring peptide and protein biomarkers** from onset and through progression of neurodegenerative diseases, at µg/L levels and below in small µL sample volumes.
4. **To characterise the uptake, metabolism and transport to the brain of metals and metal containing biomolecules** related to neurodegenerative diseases using the developed methods and spike materials. In addition, to develop accurate methods for the quantification of metals and the

co-localisation of metals with biomarkers relevant for neurodegenerative diseases in biological samples.

5. **To facilitate the uptake of the technology and measurement infrastructure** developed by the project by the measurement supply chain (accredited laboratories, instrumentation manufacturers), standards developing organisations (ISO, CEN) and end users (medical practitioners, medical (academic) hospitals and industry).

Progress beyond the state of the art

A definite diagnosis of AD is only possible post-mortem. This is due to the symptomatic overlap between different forms of neurodegenerative diseases. While diagnosis in patients is commonly achieved via cognitive tests and brain imaging, unambiguous AD diagnosis requires post-mortem histopathological verification of brain atrophy alongside β-amyloid (aβ) plaques and tau-protein based neurofibrillary tangles. The cerebrospinal fluid (CSF) which surrounds the brain and the spinal cord is more accessible than brain tissue. However, CSF is extracted via a lumbar puncture which is more invasive than blood collection and is only permitted if no other means to verify a diagnosis are available. Therefore, AD biomarkers in blood-derived samples are highly desirable and considerable research efforts are underway to identify the most significant biomarkers.

As a result of the project, methods have been developed which enable the quantification of the biomarkers in CSF, blood-derived samples (e.g. serum) and in brain tissue. The relationship between the biomarkers extracted from different sources has also been investigated. The biomarkers investigated included the most commonly used AD biomarkers; A β1-40, A β1-42, T-tau and P-tau; as well as metalloproteins such as the iron (Fe) containing proteins transferrin (TRF) and ferritin (FER)), copper (Cu) bound to albumin (Cu-ALB), and the Cu containing proteins superoxide dismutase (SOD1) and ceruloplasmin (CER). Additionally, metals which are suspected to be involved in the formation of tangles and plaques were assessed within this project. Brain tissue, serum, as well as artificial and human CSF were used as model samples for method development, and special focus was placed on handling, preparation and storage of the samples with the aim to identify potential changes in concentration or structure of the relevant biomarkers that can occur during and after sampling.

This project has advanced the state of the art by providing potential reference measurement procedures for most of the biomarkers named above using isotope dilution (ID) analysis in combination with either mass spectrometry (MS) or Raman spectrometry. Furthermore, amino acid analysis and determination of total phosphorus (P) and sulphur (S) content in tau-protein has been applied. Different methods for the determination of phosphorylation pattern of tau-proteins have been evaluated.

Results

Development of methods for the traceable quantification of metals and metal containing biomolecules

This objective was successfully completed, potential reference measurement procedures based on species specific IDMS were developed for metals and metal containing biomolecules.

For TRF, SOD1, CER and Cu-ALB methods suitable for the low concentrations required for their determination in CSF and brain tissues have been developed. Target uncertainties < 15 % were achieved in clinical samples (serum, CSF and brain tissue) for the determination of SOD1, TRF and Cu-ALB. Feasibility of the developed species specific IDMS was proven also for CER and Fe bound to FER. Their validation could not be carried out within the lifetime of the project. In general, the determination of FER via S or Fe presented the biggest challenge. The preservation of FER in its native form and the presence of other interfering proteins, mainly ALB, hampered its quantification in serum and CSF. Furthermore, it is difficult to quantify this protein via its metal content alone, taking into consideration the varying number of Fe atoms stored within the protein (up to 4000). To address this, PTB is currently investigating a completely different approach using FER specific peptides after tryptic digestion.

In case of HGB it turned out that this protein is not present in a free form in CSF but bound to haptoglobin which makes it impossible to transfer the method developed in the previous EMRP project HLT05 to CSF. First standard operating procedures (SOPs) have been published on the quantification of the mentioned metalloproteins in plasma, CSF and brain homogenate. Stability studies of the investigated metalloproteins in

the various matrices were performed and the information about the storage and handling conditions was included in the SOPs.

In addition to metalloproteins, metal ions such as Fe, zinc (Zn), Cu, aluminium (Al) and lead (Pb) are suspected to be directly or indirectly involved in the development of AD. Up to now, the only reference measurement procedures for the determination of metals in serum listed in the JCTLM database are for Cu, Zn and Pb, with LOQs being too high for quantifying the low concentrations expected in brain tissue and CSF. Within this project potential reference measurement procedures for the above-mentioned metals in serum, CSF and tissue with results traceable to the SI have been developed including various microwave assisted digestion methods requiring only small sample volumes. The developed measurement procedures were then applied in interlaboratory comparisons for the quantification of the metals in CSF, serum and brain homogenate. For the brain digest the results are in good agreement and for most of the results in serum. For CSF samples, which were contained in different vials, variable trace elemental backgrounds seem to be an issue, leading to disagreeing values of critical elements such as e.g. Zn among the different partners. However, the application of orthogonal methods to the quantification of trace elements in CSF of the same vial delivered excellent methodological agreement. For Al and Pb the concentrations were too low in the CSF, serum and brain samples to be quantified reliably.

Production and characterisation of isotopically labelled spike materials

This objective was successfully completed, natural and isotopically labelled materials were prepared and fully characterised to be further used for the development of the SS-IDMS methods.

Pure proteins were purchased and characterised regarding their identity, purity and metal content to be used as calibration materials. Spike materials of the metal containing proteins were produced in-house by exchanging the natural metal with an isotopically enriched one and the resulting labelled proteins were characterised accordingly. Three of the isotopically labelled metalloproteins were produced for the first time: recombinant FER labelled in ^{34}S (^{34}S-FER), native FER in ^{57}Fe (^{57}Fe-FER) and native ALB with ^{65}Cu (^{65}Cu-ALB). 5,5′-Dithiobis-2-nitrobenzoic acid (DTNB), which is used as Raman active marker, was purchased and characterised both with natural isotopic composition and the isotopic enriched form. For aβ1-40 and aβ1-42 both the natural and the isotopically labelled peptides have been characterised by peptide impurity corrected amino acid. Both the references and the spike materials are now ready for use.

Development of new and accurate methods for measuring peptide and protein biomarkers

This objective was successfully completed, methods for two aβ peptides and protein biomarker, T-tau, were developed.

Methods for two aβ peptides were developed and validated that enables the determination of these peptides in clinical samples such as CSF at the relevant physiological µg/L levels and in small µL sample volumes. The new measurement procedure for T-tau based on a SERS immunoassay sandwich approach was developed with a sensitivity sufficient for clinical samples. For this, a complex of ‘gold nanoparticles (gNP) – 5,5′-Dithiobis-2-nitrobenzoic acid (DTNB) – monoclonal anti-tau antibody – tau protein – polyclonal antibody – magnetic nanoparticle (mNP)’ was successfully prepared for both with the DTNB with natural isotopic composition and the isotopic enriched form. Since the total hybrid complex is too stable to aggregate, which would be important for the measurements with surface enhanced Raman spectrometry (SERS), new measurement procedures were investigated. However, as the method development took longer than expected the method validation in CSF at physiological levels is still in progress. For the determination of T-tau and P-tau, various methods for the removal of interfering matrices have been investigated using precipitation or immunoprecipitation. The most promising approach has been developed further in order to improve the enrichment to the factor needed for the low concentrations of phosphorylated proteins in CSF or brain matrix. For P-tau there were too many interferences in clinical samples to quantify this biomarker reliable via phosphorylated peptides.

Uptake, metabolism and transport to the brain of metals and metal containing biomolecules

This objective could only be partly achieved. The methods for metals and IR of the metals could developed and were applied to the mouse models. However, further investigations for the determination of the metal IR in specific metalloproteins are still required to achieve the required complete isolation of these proteins from the biological matrix.

Besides the determination of metalloproteins and metal ion contents, metrologically validated procedures for the determination of the isotopic composition of the metals in plasma, CSF and brain tissue have been

developed. The special challenge, beside the separation from the complex matrix, are the low sample volumes available. A method for the determination of Fe and Cu isotope ratios at ultra-trace level could be developed that requires only very small sample volumes. The methods for the determination of metals and isotope ratios were then applied to the mouse models for tau and aβ pathology and were compared to the according wild-type mouse lines. The data revealed some statistically significant differences in isotopic compositions of Fe, Cu and Zn in the brains and serum of the tested mice lines and an indication of differences in the metal content as well, indicating a change in the processes of metal homeostasis. This proof-of-concept study opens the way for investigating whether isotopic information can be used for diagnostic purposes and/or to achieve a more profound understanding of the disease conditions. If changes are revealed this method can serve as a novel method for medical diagnosis for AD at a much earlier stage than is possible with current biomarkers and could even enable a prognosis for people at risk of developing AD.

Impact

The project partners regularly interacted with relevant stakeholders (hospitals, clinical laboratories, reference laboratories) and the project results have been disseminated through the following activities:

- Initial project results were presented at the JCTLM stakeholder workshop in December 2017 with attendees from the metrological community, reference laboratories, clinicians and IVD industry. Results from the project will be presented at the 2019 stakeholder workshop in December.
- An advisory board, consisting of researchers, a clinical reference laboratory and a member of the society Alzheimer's Research UK (ARUK) amongst others was established to ensure that the research also meets the needs of clinicians as well as clinical researchers in the field of neurodegenerative diseases.
- A stakeholder workshop was held in conjunction with the kick-off meeting in 2016 with attendees from reference laboratories, hospitals and caretakers for the consortium to understand all aspects involved in the development of AD and to discuss planned activities with the stakeholders. In addition, the project held a dedicated session during the 6th International Symposium on Metallomics in Vienna in 2017 for a mainly scientific audience.
- The ReMiND 2019 - Biomolecules in Neurodegenerative Diseases conference was organised at PTB in June 2019. 28 participants, including some high-profile speakers, from all over Europe contributed to the conference with presentations, posters and lively discussions. Participants came from research institutes, hospitals and vendors. As part of the conference, the project partners presented the measurement procedures developed within the project and also discussed future activities with the stakeholders for their implementation within the wider community and also with colleagues involved in the EMPIR project NEUROMET, another project concerned with the diagnosis of neurodegenerative diseases.
- Training material on the developed measurement procedures will be published as soon as they have been published in peer-reviewed journals. The first SOPs have already been published on the project website and some other training material is currently in production and will be available soon.
- Raising the awareness of “future” researchers about metrology and the need for it by presenting the project at several RSC organised general student and early career researcher activities (ao. Twitter conference, Analytical Science Network: Bright spark Symposium) and at conferences and meetings of student and early career researcher activities connected with ARUK (Alzheimer Research UK). This is important since metrology does not feature in the normal curriculum of either chemistry students or medicinal / neurological orientated studies.

Impact on industrial and other user communities

A European Network on Traceability in Laboratory Medicine (TraceLabMed) has been set-up. As this network brings together all relevant stakeholders in the field of laboratory diagnostics including IVD producers, clinical laboratories and medical societies, the partners will use this network for further dissemination. Furthermore, the involved NMIs and DIs will offer services based on the methods developed within this project through the network.

Producers of calibration and matrix reference materials are now able to benefit from the project. As many of them are partners of both JCTLM and TraceLabMed, they have been made aware of the methods developed

in this project via both JCTLM and TraceLabMed meetings. The developed reference measurement procedures allow them to provide reference materials with values directly traceable to the SI. Those materials are urgently needed for quality control in clinical laboratories. LGC, a potential producer of such materials, is one of the project partners.

The reliable and comparable measurement procedures developed in this project are now able to support physicians and pharmaceutical companies in the development and testing of potential treatments for AD in clinical studies. A pharmaceutical company currently working on the development of such a treatment was associated with this project providing advice as well as the stock for breading the tau mouse model. The work has already attracted the interest of a German and a Canadian university who are interested in a future collaboration in this field. They currently investigate reasons for the onset of neurodegenerative diseases and will need measurements of the biomarkers developed within this project. Besides providing measurement capabilities also training and student exchanges is currently discussed. The procedures developed within this project will not only support research in the field of AD. LGC supported a clinical study on Wilson's disease, rare genetic disorder that causes copper poisoning in the body, by applying the procedures for Cu-ALB developed within this project.

The ability to measure changes in the concentration of metals and metalloproteins as well as in their isotopic composition has provided physicians with the ability to understand the biological processes leading to AD in more detail and help to identify the right time to start treatment. Furthermore, the determination of trace element distribution between plasma and brain tissue, on the basis of isotope ratios, can provide information about uptake rate and trans-localisation of trace elements into the brain and will significantly contribute to the understanding of how the metabolism of essential trace elements is influenced during AD progression.

The activities on the quantification of FER in serum have attracted attention of a European consortium including research institutes and IVD producers currently formed to establish a more reliable determination of FER and Fe load of FER in serum in clinical routine laboratories. The first stage was positively evaluated, and a full project proposal is currently compiled.

Impact on the metrology and scientific communities

The project has provided the metrological basis for the traceable, reliable and comparable determination of established and novel AD biomarkers in biological samples. In particular, reference laboratories can benefit from the availability of SI-traceable reference values provided by the NMIs/DIs involved. These reference laboratories play a significant role as they can now use the reference values in their interlaboratory comparisons for routine clinical laboratories, thereby providing traceability for patients' samples. This makes results of the routine clinical laboratories more reliable and provides a basis for establishing universal cut-off values for the diagnosis of AD, rendering it unnecessary for every laboratory to define their own cut-off value and control group. One of the German reference laboratories was a collaborator of this project ensuring the dissemination to this community. To make other NMIs/DIs in Europe aware of the project, it was presented at the EURAMET TC-MC and CCQM meetings. PTB already offers a measurement service for SOD1.

PhD students involved in the project participated in the activities of the Alzheimer's Society funded Doctoral Training centre at UNIABDN and the Alzheimer Research UK conference. Furthermore, results of this project were presented at various key scientific conferences such as the 6th International Symposium on Metallomics and the Winter Conference on Plasma Spectrochemistry and published in key peer-reviewed journals.

Impact on relevant standards

The reference measurement procedures developed within this project will enable the implementation of the EU regulation 2017/746 on *in vitro* diagnostic medical devices which states clearly that "the traceability of values assigned to calibrators and/or control materials must be assured through available reference measurement procedures and/or available reference materials of a higher order". Moreover, the standard EN ISO 17511:2003 demands reference measurement systems including reference measurement procedures for the determination of analytes in samples of human origin. However, neither reference measurement procedures nor reference materials of higher order for analytes related to AD currently exist. One of the aims of the network TraceLabMed is to assist the parties involved in the implementation of the new EU regulation 2017/746. The results achieved in this project will serve as examples of how traceability in laboratory diagnostics can be established.

Longer-term economic, social and environmental impacts

As the risk of getting AD increases with age and people are tending to live longer, so the number of AD patients is expected to increase in the future, with estimates of up to 90 million dementia patients worldwide within the next 20 years, making dementia one of the greatest health issues today.

Besides the importance of early diagnosis to potentially develop a future cure for AD, reliable early diagnosis is important as early intervention can delay the on-set of severe symptoms of dementia, resulting in a better quality of life for AD patients and their carers. However, for such an early diagnosis, universal cut-off values to distinguish between healthy and diseased people and reliable routine measurement procedures are a prerequisite. The reference measurement procedures developed in this project will help to improve the reliability of routine measurement kits and reduce the variations between the results of different kits thus enabling the establishment of the required universal cut-off values.

Dementia is already burdening health care systems with total estimated costs of € 32.8 billion in Europe in 2008. Included in these costs are those attributed to informal care such as unpaid care provided by family and others, direct costs of social care such as community care professionals and in residential homes, and the direct costs of medical care. Direct medical care costs account for roughly 20 % of global dementia costs, while direct social sector costs and informal care costs each account for roughly 40 %. Sensitive and reliable measurements will contribute to a reduction of these health care costs because they will enable early detection of AD and, therefore, allow earlier intervention resulting in a delayed on-set of severe impairments requiring hospitalisation. As well as improving the quality of life for both patients and their families, delayed hospitalisation is estimated by some studies to save around US$ 10,000 per person with dementia over the course of the disease.

List of publications

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Project start date and duration:	01 July 2016, 36 months	
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Internal Funded Partners: 1 PTB, Germany 2 BAM, Germany 3 LGC, United Kingdom 4 TUBITAK, Turkey	External Funded Partners: 5 Charité, Germany 6 UGent, Belgium 7 UNIABDN, United Kingdom 8 UNIVIE, Austria	Unfunded Partners:
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