The Link between Salivary Surfactant-Protein D and Dementia in Nursing Home Dementia Patients

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ABSTRACT
Recently, serumal surfactant protein D (SP-D) has been shown to correlate with the development of dementia. As SP-D can be measured reliably in saliva, this might provide opportunities for a non-invasive biomarker. This preliminary study aims to determine a possible difference between the levels of salivary SP-D in non-demented elderly and late stage demented elderly. Results indicate a significant difference between demented subjects and controls, and between male and female demented subjects. This suggests SP-D could be used as a biomarker to improve detection of dementia. Further research should focus on early-stage dementia and the role of innate immunity in dementia.

Keywords
Dementia, surfactant protein D, innate immunity, saliva, biomarker

INTRODUCTION

In the search for a cure for dementia, it is of major importance to be able to detect this disorder in an early stage. Biomarkers could be especially important in clarifying the underlying pathophysiologies of dementia, such as Alzheimer's disease, and could eventually lead to improved diagnostic tools and treatment options (DeKosky & Marek, 2003). This search for new, improved biomarkers is an ongoing process. An example of a recently proposed biomarker for dementia is surfactant protein D (SP-D) (Nybo et al., 2007).

SP-D and innate immunity

SP-D is a surfactant protein and is a member of the collectin-family. It is primarily known for its pulmonary function and, as such, is involved in the host defence response and the clearance of apoptotic cells (Vandivier et al., 2002). In addition, SP-D knock-out mice showed increased aggregation of alveolar macrophages and a disturbed lipoprotein homeostasis (Botas et al., 1998). Interestingly, Schob and colleagues (2013) recently reported that SP-D was expressed in the brain of adult rats, specifically in the blood-brain barrier, choroid plexus, and superficial glia limitans. This, together with the fact that surfactant proteins regulate inflammatory responses and help clear apoptotic cells, suggest that these proteins play a role in the innate immune system of the brain and cerebrospinal fluid (Schob et al., 2017).

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Innate immunity and dementia

Recent research (Heneka, Golenbock, & Latz, 2015) has suggested neuroinflammation plays an important role in Alzheimer's disease's pathology. In general, the formation of beta-amyloid (Aβ) peptides is seen as the first (detectable) step in the development of dementia. This aggregation of Aβ could lead to chronic activation of the immune system. In turn, the immune system causes a disturbance in microglial functioning, especially with regards to clearance of apoptotic cells. In addition, Alzheimer's disease (AD) was reported to correlate with extensive upregulation of genes involved in the innate immune system, possibly even in preclinical stages (Cribbs et al., 2012), supporting the notion that the immune system plays an important role in the development of dementia.

SP-D and dementia

Nybo and colleagues (2007) examined serumal SP-D levels of 418 non-demented participants at baseline and after three years of follow-up. At follow-up, 27 of the 346 remaining participants showed signs of dementia. From the blood samples, it was concluded that higher serum SP-D levels correlated with an increased risk of developing dementia, with a risk ratio of 2.62. A strong connection between the development of dementia and inflammatory reactions has been suggested. As SP-D is known to play a role in the innate immune system, the finding that SP-D is correlated with the development of dementia could provide new insights in the underlying pathophysiology and could even lead to a new biomarker to detect dementia in an early stage.

Ideally, biomarkers to detect dementia would be inexpensive, easily collected and analysed, and minimally invasive. As SP-D has been shown to be present in saliva (Bräuer et al., 2009), the use of salivary SP-D would be preferred over the use of serum SP-D. Nevertheless, no research has looked into the correlation between salivary SP-D levels and dementia yet. Hence, this study will determine if there are differences in salivary SP-D levels between moderately to severely demented elderly and non-demented elderly, using a Luminex multiplex immunoassay. Samples were taken in the morning and the evening, to see if there is a possible diurnal rhythm in the presence of SP-D, which could influence the efficacy of the possible biomarker. If SP-D is indeed shown to correlate with dementia, this could be a step forward in the development of non-invasive detection methods for dementia.

METHODS

Participants

All participants in this study were part of a larger study concerning the effects of bright light therapy (BLT) on moderately to severely demented elderly in nursing

homes. All participants were nursing home residents and were under constant care. In this sub-study, saliva samples from 58 participants, grouped for three different time periods, in both the morning (O1, O3, O5) and the evening (A1, A3, A5), were included. Informed consent, either self-provided or provided by relatives, was obtained for all participants. Table 1 shows demographic details of all participants.

Table 1 – Demographic details of all participants

		Demented elderly	Controls
Gender (count)	female	33	11
	male	21	6
Diagnosis (count)	Alzheimer	19	
	Vascular	4	
	Mixed	11	
	Other	20	
Age (years)	Mean	85.87	79.29
	St. Dev.	7.24	8.01

Sample collection

Samples were obtained using Salivette® cotton swabs prior to ingesting food, non-water drinks, and medication, and before brushing of the teeth in the morning (07:00 – 09:00), and an hour after dinner, before drinking coffee, in the evening (19:00 – 21:00). After collection, samples were refrigerated until centrifugation, followed by aliquoting samples into Cryo cups, and were subsequently frozen at -18˚C until use. For the comparisons between the experimental (demented) and control group (non-demented), samples from O1 were used.

Luminex multiplex immunoassay

SP-D levels were determined using a Human Premixed Multi-Analyte Kit (CHI3-L1&SP-D), cat. LXSAHM-2, lot 1363794 from R&D Systems. At an earlier time point, two plates were measured, containing an eight-point three fold standard curve, two blanks, samples from the control group, and 88 experimental samples in total. Three additional plates were measured at a later time point, each containing 78 samples from the experimental group as well as standard dilutions. In order to verify consistency between these two measurements, ten experimental samples measured at the first time point repeated at the second time point.

The antibody mixture used in this experiment was provided and verified for SP-D by R&D systems. To perform the experiment, eight three-fold standard dilutions were made, from which standard curves were generated. The wells for the standard curve contained 50 µL of the standard dilutions and 50 µL of the bead solution. Wells with samples contained 25 µL of saliva, 25 µL of calibrator diluent (as provided by R&D systems), and 50 µL of bead solution. Eight samples did not contain sufficient saliva and were diluted more, which was corrected for in the calculations.

After adding the bead solution to the wells, the plates were incubated for two hours and subsequently washed three times. Afterwards, 50 µL of detection antibody solution was added and the plates were incubated for another hour. The washing step was repeated and 50 µL streptavidin PE solution was added as a reporter protein. After incubation of 30 minutes, the plates were washed and samples were suspended in 100 µL washing buffer. The plates were incubated for two minutes and analysed using a Bioplex reader. The Bioplex reader measured the beads using two laser beams of different frequencies, the first of which identifies the bead's colour to classify the analyte. The second laser determines the fluorescence of the bead, which is an indicator of the amount of bound streptavidin PE, and thus of SP-D present on the bead. The needed time per sample was approximately thirty seconds.

Data analysis

Before statistical analysis, as per convention, samples registering as out of range on the lower bound were assigned half of the lowest measured value, as not to lose data. Additionally, the measured values were converted into their base ten logarithm, in order to reduce variance in the data.

Using a Shapiro-Wilk test, the normality of the sample was assessed. For the control group (p=.002) and most of the experimental categories (O1: p=.101, A1: p=.000, O3: p=.000, A3: p=.000, O5: p=.287 , and A5: p=.001) the null-hypothesis was rejected and therefore normality cannot be assumed for any data. Thus, these data were analysed using nonparametric tests.

First, background checks were performed. Two sets of samples were measured at two different time points, differing about a year. Control samples were only measured at the earlier time point. To check for sample and assay degradation, some previously measured samples (n=10) were assayed again. Subsequently, their data were compared using a Wilcoxon signed-rank test. In order to rule out differences in age and sex between the control group and experimental group as confounding factors, a Mann-Whitney U test and a Pearson's χ^2-test were used, respectively. To assess the main research question and sex differences in the logarithm of the observed concentrations, Mann-Whitney U tests were used to identify significant differences in means. A Wilcoxon signed-rank test was used to identify a possible diurnal rhythm of SP-D. A Kruskal-Wallis H test was used to assess the differences in SP-D concentration across the different diagnoses (Table 1). Lastly, to assess the relationship between SP-D concentration and age, a bivariate analysis using Pearson's r was used.

RESULTS

Background checks

The earliest measured samples (M=2.0504, SD=.6303) were significantly different (Z=-2.599, p=.006) from samples measured at the later time point (M=2.3342, SD=.3116). The gender distribution did not differ by experimental condition, $\chi^2(1, N=71)=.071$, p=.790. A Mann-Whitney U tests showed that there was a significant difference in age between the experimental (M=85.87, SD=7.237) and control group (M=79.29, SD=8.006), (Z=-2.996, p=.003).

Controls versus dementia patients

Concerning the main research question, a Mann-Whitney U test showed there is a large, and highly significant difference between the logarithm of the observed SP-D concentration in the experimental (M=2.3883, SD=.6641) and control group (M=1.2037, SD=.6975), (Z=-4.530, p=.000), see Figure 1.

Age and SP-D

There was no significant correlation between age and SP-D in the experimental group, $r(54)=.158$, $p=.255$, nor in the control group, $r(17)=.385$, $p=.127$.

Sex and SP-D

A Mann-Whitney *U* test showed a significant difference between male and female subjects in the logarithm of the observed SP-D concentration for the experimental group ($M_f=2.5703$, $SD_f=.5867$, $M_m=2.1023$, $SD_m=.6910$), ($Z=-2.342$, $p=.019$). However, the control group did not show a significant sex difference ($M_f=1.1921$, $SD_f=.8687$, $M_m=1.2249$, $SD_m=.9544$), ($Z=-.208$, $p=.884$), see Figure 1. This suggests an effect of sex on salivary SP-D levels in dementia patients, but not in the non-demented elderly. However, the small size of the control group might explain the lack of significance.

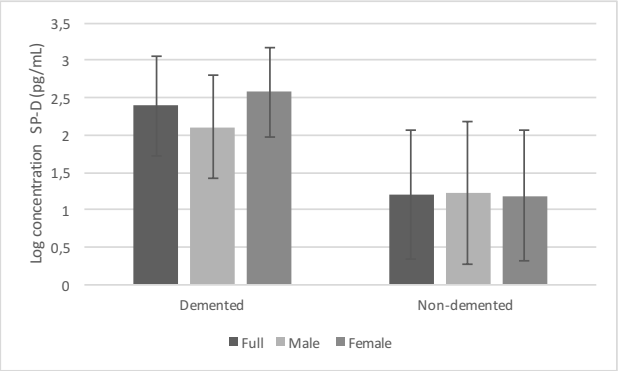

Figure 1 - Logarithm of observed concentration of SP-D in non-demented and demented elderly; overall averages and by sex. This graph illustrates the significant differences between demented and non-demented elderly ($p=.000$), and between males and females in demented elderly ($p=.019$) as opposed to in non-demented elderly ($p=.884$).

Diagnosis and SP-D

There was no significant difference between the logarithm of the observed concentration for patients with different diagnoses ($H(3)=2.128$, $p=.546$), with a mean rank of 24.9 for Alzheimer's Disease, 20.5 for vascular dementia, 29.3 for mixed diagnoses, and 30.4 for undefined diagnoses, suggesting that diagnosis and SP-D levels in demented elderly do not correlate.

Diurnal rhythm and SP-D

Wilcoxon signed-rank tests showed that there is a significant difference in the logarithm of the SP-D concentration between morning and evening samples for day one and three ($Z=-3.405$, $p=.001$; $Z=-2.669$, $p=.007$), but not for day five ($Z=-1.599$, $p=.113$). These data, especially in combination with the similarity of the differences in means ($\Delta M_1=.26$, $\Delta M_3=.23$, $\Delta M_5=.21$), suggest a probable diurnal rhythm for SP-D in elderly with dementia. The lack of significance on day five could be explained by the smaller sample size as an effect of higher mortality.

DISCUSSION & CONCLUSION

The fact that the differences between SP-D concentrations in controls and dementia patients were significant suggests that salivary SP-D concentrations are higher in dementia patients. This supports the hypothesis that SP-D plays a role in dementia, even though the mechanism behind it is still unknown. Part of this effect might be explained by the significant difference between the sample sets measured at the different time points, as the control samples were all measured at the earlier time point and on one plate. However, the difference between the two repeated measurements ($\Delta M=.2383$) is much smaller than the difference between the control and experimental groups ($\Delta M=1.1846$). This suggests that there is still a difference in SP-D concentration between non-demented and demented elderly, yet the results should be treated with caution. Additionally, the significant difference between morning and evening samples indicates the presence of a diurnal rhythm for SP-D. Lastly, the significant difference in SP-D concentrations between the sexes in demented elderly could point at possibly sex-specific underlying mechanisms of SP-D. Due to small sample size, however, this effect could not be verified in control subjects.

The higher SP-D concentration in dementia patients could be an indicator of an innate immune system response to a chronic inflammatory reaction in the brain (Heneka et al., 2015). SP-D has been shown to have both pro- and anti-inflammatory properties (Gardai et al., 2003), so it could either enhance this reaction, or reduce it by enhancing clearance of apoptotic cells. This clearance might be disturbed in the brain of dementia patients and thus contribute to the development of dementia (Kao et al., 2011). In addition, SP-D might be associated with microglia, which have similar clearance functions in the innate immune system (Heneka et al., 2015). Therefore, further research should try to clarify the exact relationships between SP-D, the innate immune system, and the development of dementia.

These results support the suggestion that SP-D is a good candidate for a biomarker for dementia detection, as well as a subject for future research into the pathophysiologies underlying dementia. The fact that there were no significant differences in concentration across the diagnoses suggests SP-D could detect multiple types of dementia, however not distinguish between these, and could thus be used in a wide variety of clinical and research settings. A simple saliva collection procedure could, in the future, possibly become part of a swifter and safer diagnosis process without an immediate need for neuropsychological testing.

As our preliminary study was part of a bigger study, the experimental group consisted mostly of moderately to severely demented elderly. This means this study can be used as a proof of principle, but that to adequately assess the use of SP-D as a biomarker for dementia, future studies should focus on mildly demented subjects or on birth cohorts of risk-populations. The sample sizes in this study, specifically for the control group, were relatively small, suggesting that the results might not be generalisable to the entire population. In addition, there was a significant difference in age between the control and experimental group, which should, however, not affect this study's conclusions, as age was not significantly correlated with SP-D concentration. Furthermore, the samples were not all measured at the same time, which led to a significant difference between the observed concentrations measured at different time points, yet as described above, this should not change the main conclusions of this study. Lastly, many values were out of range at the lower bound and could therefore not be determined exactly. This was corrected for by

taking half of the lowest value, which might have affected the exact findings of this studies, but most likely not its conclusions.

To adequately assess the relationship between SP-D and dementia, future studies should use larger sample groups and age-matched controls. Such a bigger study should include a wide range of subjects with several types of dementia. Only such a study could appropriately verify if there are indeed no significant differences in SP-D concentrations across the diagnoses, and if there is no sex difference in non-demented elderly. Additionally, such a study could also analyse the concentration differences separately for both sexes in order to rule out a sex-effect, where SP-D would be a reliable biomarker in one sex, but not the other. Secondly, a cohort study in elderly, or a population-wide study, is recommended to appropriately assess the use of SP-D as a biomarker and predictor for dementia, as well as to identify effects of covariants, such as age and sex, on SP-D levels across all ages. Such a study could also determine if SP-D could be used to differentiate between other neurodegenerative disorders, such as Parkinson's disease, and the several types of dementia. Lastly, as SP-D levels were found to be relatively low, it is recommended to use a more sensitive detection method to determine these concentrations of SP-D, or alternatively, repeat measurements of out of range values with a doubled sample volume.

In conclusion, this preliminary study serves as a proof of principle that salivary SP-D is a promising candidate to serve as a biomarker for detection of dementia and could provide new insights the underlying pathophysiology of dementia. To confirm these findings and to identify the exact relationship between dementia, SP-D, age and sex, future research is needed.

ROLE OF THE STUDENT

Céline Budding and Toon Holman were undergraduate students working under the supervision of dr. Gerda Andringa and prof. Ger Rijkers when the research in this report was performed. The topic was proposed by the supervisor. The Luminex assays were performed by Ben de Jong, a lab technician in the St. Antonius Ziekenhuis in Nieuwegein, the Netherlands. The design of the experiment, the processing of the results as well as formulation of the conclusions and the writing were done together by the students.

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