Neutrophil Extracellular Traps: A Perspective of Neuroinflammation and Complement Activation in Alzheimer’s Disease

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Complement system (CS) components are associated with Alzheimer’s disease (AD), the commonest cause of dementia in the world. Neutrophils can be attracted to amyloid-β plaques by several pro-inflammatory factors, including the complement anaphylatoxin C5a. They may release neutrophil extracellular traps (NETs), which are chromatin nets associated with myeloperoxidase, elastase, and other enzymes. Some CS molecules, such as C5a, C1q, and CR1, are associated with increased neutrophil recruitment and NETs release. However, the relationship between CS molecules and NETs in AD is poorly understood. In this work, we detected higher NET concentrations in plasma and serum of Brazilian AD patients, than in elderly controls (medians = 2.78 [2.07–6.19] vs. 2.23 [0.33–4.14] ng/mL, p = 0.0005). We discussed these results within the context of our former findings on complement and AD and the context of the literature on complement and NET release, suggesting both as possible therapeutic targets to prevent the progress of the disease.

Keywords: Alzheimer’s Disease, neutrophil extracellular traps, inflammation, complement system, CR1, C5a, C1q

INTRODUCTION

Neuroinflammation is a well-established phenomenon in AD (Czirr and Wyss-Coray, 2012; Wyss-Coray and Rogers, 2012; Dansokho and Heneka, 2018; Kloske and Wilcock, 2020) whose mechanisms are still poorly understood. They are related to the accumulation of amyloid-β (Aβ) plaques and neurofibrillar tangles (NFTs), characteristic AD biomarkers. The first present damage-associated molecular patterns (DAMPs) (Wyss-Coray and Rogers, 2012; Heppner et al., 2015) which are recognized by the complement system (CS) (McGeer et al., 1989; Veerhuis et al., 2011; Tenner et al., 2018). They also induce the expression of endothelial adhesion molecules and the release of pro-inflammatory cytokines by stimulated glial cells (reviewed in Pietronigro et al., 2017). Indeed, the CS appears to play a relevant role in AD, as judged by the strong association of complement genetic polymorphisms with this disease (Morgan, 2018; Kranze et al., 2019; Kretzschmar et al., 2020; Tenner et al., 2020). Besides that, the CS has already been correlated with the formation of neutrophil extracellular traps (NETs) (Palmer et al., 2016; de Bont et al., 2019). NETs are composed of chromatin fibers, citrullinated histones, and cytoplasmic enzymes as myeloperoxidase (MPO) and...
neutrophil elastase (NE), which altogether operate as an extracellular platform for trapping and killing bacteria (Brinkmann et al., 2004; Yousefi et al., 2009). They have been observed in AD patients and an AD animal model (Zenaro et al., 2015; Dong et al., 2018). However, the possible role of the CS in NET release within AD has never been discussed before. In this study, we focused on reported interactions of the CS with Aβ plaques and their possible role in neutrophil recruitment and NETs release in AD. We also confirmed the presence of higher NET levels in an AD Brazilian cohort.

**ALZHEIMER’S DISEASE AND THE COMPLEMENT SYSTEM**

AD is a neurodegenerative disease responsible for the largest number of dementia cases globally (Alzheimer's Association, 2020). Several authors sought to understand the disease's etiology by analyzing different pathways and metabolic processes, most of them causing or being influenced by neuroinflammation (Zlokovic, 2011; Heppner et al., 2015; Du et al., 2018). In AD, the production of Aβ plaques and NFTs are exacerbated. The accumulation of Aβ plaques in the extracellular environment causes the loss of interneural communication (synapses) and activates the local and systemic immunological responses since Aβ plaques can be recognized as DAMPs by phagocytic cells (Czirr and Wyss-Coray, 2012; Wyss-Coray and Rogers, 2012; Heppner et al., 2015) and activate the CS (McGeer et al., 2016).

The CS is considered a key element in innate immunity, playing an essential role in regulating and protecting the central nervous system. The CS consists of an enzymatic cascade with the participation of more than 50 circulating proteins, as well as soluble or membrane receptors and regulators (Lee et al., 2019). It can be activated by three different pathways: classical (CP), lectin (LP), and alternative (AP). For more information on activation and participating CS elements, consult the review of Ricklin et al., 2016. Although the complement pathways are activated in different ways, they all enhance phagocytic activity and may progress to the formation of membrane attack complexes (MAC), leading to cell lysis. In addition, the activation of the complement cascade also results in anaphylatoxin production and recruitment of inflammatory cells. The CS must be tightly regulated. If out of control, the cascade may become offensive, permanently injuring surrounding tissues (Ricklin et al., 2016).

It is not a novelty that CS components and genetic polymorphisms are associated with AD (McGeer et al., 1989; Webster et al., 1997; Loeffler et al., 2008), as complement component 3 (Stoltzner et al., 2000; reviewed by Wyss-Coray and Rogers, 2012; Goetzl et al., 2018), complement component 4 (reviewed by Wyss-Coray and Rogers, 2012; Goetzl et al., 2018), complement membrane complex C5b-C9 (Goetzl et al., 2018), complement component 3α (C3α) and its receptor C3αR (Lian et al., 2015; Litvinchuk et al., 2018), complement component 5α (C5α) and its receptor C5αR1 (An et al., 2018), complement receptor 1 (CR1) (Lambert et al., 2009; Kretzschmar et al., 2020), clusterin (CLU) (Lambert et al., 2009) and complement component 1q (C1q) (Stoltzner et al., 2000; reviewed by; Wyss-Coray and Rogers, 2012; Lian et al., 2016; McGeer et al., 2016; Dejanovic et al., 2018; Goetzl et al., 2018), complement component 9 (reviewed by Wyss-Coray and Rogers, 2012), factor B and factor D (Goetzl et al., 2018). In this work, we will focus only on the CS components associated with AD, which may be related to the recruitment of neutrophils and the formation of NETs (Figure 1).

**ALZHEIMER’S DISEASE AND NEUTROPHIL TRAPS**

Neutrophils have an essential role in inflammation, acting through many mechanisms: phagocytosis, degranulation, and extrusion of NETs (reviewed by Shen et al., 2001; Rossi et al., 2020). NETs are involved in host tissue injury and inflammation associated with autoimmune diseases, acute injuries, atherosclerosis, vasculitis, and cancer (Jorch and Kubes, 2017). In 2015, Zenaro and colleagues observed NETs adjacent to Aβ plaque deposits in the cerebral vascular and intraparenchymal region of an AD animal model and European AD patients. They suggested that Aβ plaques may play an essential role in the recruitment and movement of neutrophils, which Baik et al. (2014) also observed in an AD animal model. Furthermore, NET extrusion was also detected in high concentrations in European AD patients’ serum (Dong et al., 2018).

**C5α-C5αR1 Axis and the Relation With Neutrophil Traps and Alzheimer’s Disease**

After complement activation (McGeer et al., 1989; Veerhuis et al., 2011), C3 and C5 are cleaved, generating the anaphylatoxins C3α and C5α (reviewed by de Bont et al., 2019). C3α lacks chemotactic activity (Ehrengruber et al., 1994), but C5α generates a potent chemotactic response and induces neutrophil migration (Ehrengruber et al., 1994), as well as the release of NETs (reviewed by de Bont et al., 2019). Therapeutic inhibitors blocking C5a and/or its receptor C5αR1 have been proposed to treat AD (Fonseca et al., 2013; Landlinger et al., 2015; An et al., 2018). Although the functional connection between high NET levels and C5α has been well described (Yousefi et al., 2009; Huang et al., 2015; Yuen et al., 2016; Fattahi et al., 2018; de Bont et al., 2019), it has never been explored in AD.

The CS seems to be, at least through C5a, attracting neutrophils to the brain and inducing NETs extrusion. Neutrophil activation by C5a extrudes the mitochondrial DNA (Yousefi et al., 2009), which suggests that in AD, after NETs extrusion, the neutrophils do not die, at least not through C5a stimulation. NETs deposition triggers CS activation via the alternative pathway and properdin binding (Yuen et al., 2016). The neutrophils probably recognize the Aβ plaques and trap them, resulting in increased inflammation and tissue injury. After triggering the neutrophils to the tissue, the CS may not degrade the NETs, resulting in tissue NET accumulation and CS over-activation (de Bont et al., 2019). Thus, the use of C5α inhibitors may decrease NETs activation in AD.

**CR1 and Its Relationship With Neutrophil Traps and Alzheimer’s Disease**

Even before NET’s discovery, several authors sought to understand the mechanisms of interaction between the complement system and...
neutrophils. Cytokines as tumor necrosis factor-alpha (TNFα), granulocyte-monocyte and granulocyte colony stimulating factors (GM-CSF, G-CSF), interleukin 1 (IL-1), platelet activating factor (PAF), and lymphotoxin-beta (LTB) up-regulate phagocytic complement 1 receptor (CR1, also known as CD35) in neutrophils, increasing its association with C3b-opsonized microspheres. However, only TNFα, G-CSF, and PAF increased their phagocytic uptake (Ogle et al., 1990). Besides that, CR1 recognizing C3b-IgG complexes on the
neutrophil membrane led to changes in signal transduction events associated with Fc receptors, resulting in myeloperoxidase’s release and generation of hypochlorous acid (Sambandam and Chatham, 1998). CR1 blockage is followed by a decrease in NET concentration, revealing an essential role of this molecule in the extrusion process (Palmer et al., 2012). Thus, recognition of C3b-opsonized particles by CR1 on neutrophil membranes may preferentially lead to the release of NETs instead of phagocytosis, although more studies are needed to corroborate this hypothesis.

Interestingly, increased AD susceptibility has been repeatedly associated with CR1 polymorphisms (Lambert et al., 2009). Nowadays, the hypothesis proposed for this association is that some polymorphisms (such as rs6656401*A) facilitate non-homologous recombination resulting in the preferential expression of dysfunctional CR1*B isoform (Brouwers et al., 2012; Mahmoudi et al., 2018). sCR1 is a potent local inhibitor of the complement system and is expressed in lower amounts in erythrocytes than the CR1*A functional protein (Mahmoudi et al., 2018). Thus, CR1*B probably impairs the process of removing Aβ plates and regulating the CS (Mahmoudi et al., 2018). Heterozygote CR1*A/CR1*B individuals express both isoforms. In this case, neutrophil recognition of C3b-opsonized Aβ plates may occur by CR1*A, with consequent release of NETs. CR1 can also be found in a soluble form (sCR1). AD patients have higher levels of sCR1 in serum (Mahmoudi et al., 2018), plasma (Kretzschmar et al., 2020), and cerebrospinal fluid (CSF) (Daborg et al., 2012). sCR1 is a potent local inhibitor of the complement system and is formed through vesiculation or proteolysis of the membrane-bound CR1 (Pascual et al., 1993; Danielsen et al., 1994; Dervillez et al., 1997; Hamer et al., 1998), inhibiting the CS by dissociating C3 convertases, and targeting C3b and C4b for degradation (Zhu et al., 2015). It is possible that large sCR1 quantities would inhibit complement’s beneficial role of removing Aβ plaques, contributing to its accumulation. However, no studies to date demonstrated whether individuals who have only the CR1*B isoform present functional sCR1. If sCR1 is generated from CR1*B, CS inhibition probably will not occur properly, recruiting more neutrophils to the affected region, with higher extrusion of NETs, ultimately contributing to chronic neuroinflammation. Although CR1’s participation in the increase of NETs release seems to be evident, it is not yet clear how this may be related to the different isoforms of the molecule and its association with AD. Still, it raises an exciting possibility of a new role for CR1 in the disease, which needs to be investigated in further functional studies.

### C1q and the Relation With Neutrophil Traps and Alzheimer’s Disease

The C1q molecule of the CP participates within an essential process in brain homeostasis. In periods when synapse pruning happens, C1q tags inappropriate connections between neurons for removal by the microglia (Presumey et al., 2017). In neurodegenerative diseases, C1q may lead to aberrant synapse loss (Dejanovic et al., 2018). Curiously, Aβ binds and activates C1q in the absence of immunoglobulins (Rogers et al., 1992), starting the CP and probably promoting synapse loss. A study with a mouse model of AD lacking C1q demonstrated a significant reduction in inflammation and neuropathological features (Presumey et al., 2017).

Some researchers already analyzed the relationship between C1q and NETs. Increased C1q deposition inhibits DNase activity, resulting in NET accumulation (Lefler et al., 2012). When C1q is inhibited, the complement cascade does not progress, and NETs do not appear (Hair et al., 2018). NETs are mainly degraded by endonuclease DNaseI (Hakkim et al., 2010) and then cleared by macrophages (Farrera and Fadeel, 2013). DNases have already been used to successfully treat AD in a case report (Tetz and Tetz, 2016). Genetic DNASEI variants have been investigated in systemic lupus erythematosus (Pruchniak et al., 2019), however, its role in AD has never been investigated. DNase has been used as an efficient drug to degrade NET structure in breast cancer, lung injury, and lupus mouse models (reviewed in Jorch and Kubes, 2017).

### High Neutrophil Traps Levels in a Brazilian Cohort

Recently, we confirmed the genetic association of complement receptor 1 (CR1) polymorphisms in an AD Brazilian cohort (Kretzschmar et al., 2020). Based on the association between molecules of the complement system and NETs, we aimed to investigate if NET levels are also increased within the same Brazilian cohort. We quantified NETs in plasma of 22 AD patients and 20 elderly controls (EC), and serum of another 11 EC (considering that NET levels are similar in serum and plasma of the same individual (Abrams et al., 2019)). The study was approved by the local ethics committee (CAAE 55965316.1.0000.0102). All participating individuals were older than 65 years (AD median = 82.5 [70–88] years old; EC median = 76 [69–99] years old). AD were recruited from the Clinical Hospital of the Federal University of Paraná. AD and EC were diagnosed or confirmed to be neurologically normal based on clinical history and cognitive tests (Frota et al., 2011). Diabetes and systemic arterial hypertension (SAH) are common pathologies in the elderly that may cause NET release (Soongsathitanon et al., 2019; Parackova et al., 2020). Both diseases were not associated with NETs in our study and the association of Alzheimer’s with NETs was also independent of HAS (OR = 95%CI = 1.75–6.91, p = 0.003) (data available in Supplementary Table S1). The NE-DNA concentrations in serum and plasma samples were quantified using an adapted ELISA test with immunofluorescence (Czaikoski et al., 2016; Colón et al., 2019) (Supplementary Figure S1). Anti-elastase antibodies were used for capturing these NE-DNA complexes, and dsDNA fluorescent reagent was used for detection and quantification. The data was tested for normality (D’Agostino and Pearson test). We compared patients and controls in two ways: 1) using the absolute values (ng/mL), and 2) establishing the 3rd quartile in controls as a threshold for defining high and low NET levels (2.548 ng/mL). We also used this threshold for defining the theoretical median in the Wilcoxon test. The groups were compared using unpaired T-test and two-way ANOVA. All the analyses were done using GraphPad Prism v6 software. The p-values were corrected for multiple testing using the false discovery rate (FDR) method (Benjamini and Hochberg, 1995), performed in R language 3.6.1, through the Stats package (R Development Core Team, 2008).
2011). Corrected \( p \)-values lower than 0.05 were considered significant. The data used is available in the Supplementary Table S1.

Our study brings, for the first time, higher circulating NET levels in AD Brazilian patients. In this work, we detected higher NET concentrations in plasma and serum of Brazilian AD patients, than in elderly controls (medians = 2.78 [2.07–6.19] vs. 2.23 [0.33–4.14] ng/mL, \( p = 0.0005 \) (Figure 2A). The difference remained after dichotomizing AD and EC into high- and low-NET producers. The median NET concentration of high-NET producers was 3.95 (3.09–6.19 ng/mL) in AD, compared to 3.0 (2.586–4.14 ng/mL) in EC \(( p = 0.012 \) ). For low-NET producers, the median level of AD was 2.28 (2.07–2.46 ng/mL), compared to 2.0 in EC (0.33–2.46 ng/mL) \(( p = 0.042 \) ) (Figure 2B). Although NETs extrusion can lead to cell death by NETosis, we did not perform the assays to evaluate it. Despite the small number of samples used here, few studies investigating NETs in AD patients were published. All of them confirm the increased NETs in AD (Zenaro et al., 2015; Dong et al., 2018).

## CONCLUDING REMARKS

NETs seem to be promising as new therapeutic targets for AD treatment. We propose more investigations into the connection between C5a, C1q, and CR1 with NETs in AD, as well as genetic associations studies to investigate variants in DNase genes (DNASE1, DNASE2, and DNASE1L3) that can result in a down-regulation of DNase expression in AD.

## DATA AVAILABILITY STATEMENT

The original data presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Ethics Committee of the Health Sciences Sector (Federal University of Paraná) (CAAE: 55965316.1.0000.0102), according to Resolution 466/2012 of the National Health Council and the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

AB, LD-M, and GK contributed to the conception of the work. AB, MG, VB-B, and GK designed the study. RS provided the samples. LD-M made available the infrastructure and material for the ELISA assay. MG, YZ, and KY performed ELISA assay. AB, VB-B, and GK did the statistical analysis. GK and MG made the illustrations. AB, MG, VB-B, and GK drafted the manuscript. All authors revised the work critically for intellectual content and approved the final version of the work.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2021.630869/full#supplementary-material.
Lian, H., Livvinchuk, A., Chiang, A. C.-A., Aithmitti, N., Jankowsky, J. L., and Aithmitti, N. (2018). Inherited and acquired decrease in complement receptor 1 (CR1) density on red blood cells associated with high levels of soluble CR1 in Alzheimer’s disease. Int. J. Mol. Sci. 19, 2175. doi:10.3390/ijms19081275

Mahmoudi, R., Feldman, S., Kisserli, A., Duret, V., Tabary, T., Bertholon, L.-A., Parackova, Z., Zentsova, I., Vrabcova, P., Klocperk, A., Sumnik, Z., Pruhova, S., et al. (2020). Complement activation and cognitive loss in Alzheimer’s disease. J. Neuroinflammation 5, 1–8. doi:10.1186/s12974-020-01553-w

Mahmoudi, R., Kisserli, A., Cole, A., Sun, L., Chiang, A. C.-A., Fowler, S. W., et al. (2015). NFκB-activated astroglial release of complement C3 compromises neuronal morphology and function associated with Alzheimer’s disease. Neuron 85, 101–115. doi:10.1016/j.neuron.2014.11.018

Mahmoudi, A., Wan, Y.-W., Swartzlander, D. B., Chen, F., Cole, A., Propson, N. E., et al. (2018). Complement C3aR inactivation attenuates tau pathology and reverses an immune network deregulated in tauopathy models and Alzheimer’s disease. Neuroreport 100, 1337–1333. doi:10.1016/j.neuro.2018.10.031

Litvinchuk, A., Feldman, S., Kisserli, A., Duret, V., Tabary, T., Bertholon, L.-A., Parackova, Z., Zentsova, I., Vrabcova, P., Klocperk, A., Sumnik, Z., Pruhova, S., et al. (2020). Complement C3aR inactivation attenuates tau pathology and reverses an immune network deregulated in tauopathy models and Alzheimer’s disease. J. Neuroinflammation 5, 1–8. doi:10.1186/s12974-020-01553-w

Pietronigro, E. C., Della Bianca, V., Zenaro, E., and Constantin, G. (2017). NETosis and Alzheimer’s disease: A case report. Front. Immunol. 12, 383. doi:10.3389/fimmu.2020.00661

Wyss-Coray, T., and Rogers, J. (2012). Inflammation in Alzheimer disease–A brief review of the basic science and clinical literature. Cold Spring Harb. Perspect. Med. 2, a006346. doi:10.1101/cshperspect.a006346

Shen, Y., Yue, L. F., Yang, L. B., Roher, A., Kuo, Y. M., Strohmeyer, R., et al. (2001). Complement activation by neurofibrillary tangles in Alzheimer’s disease. Neurosci. Lett. 306, 165–168. doi:10.1016/s0304-3940(01)01842-0

Soongthanasin, J., Umsa-Ard, W., and Thongboonkerd, V. (2019). Proteomic analysis of peripheral blood polymorphonuclear cells (PBMCs) reveals alteration of neutrophil extracellular trap (NET) components in uncontrolled diabetes. Mol. Cell. Biochem. 461, 1–14. doi:10.1007/s11010-019-03583-y

Stoilzner, S. E., Grenfell, T. J., Mori, C., Wisniewski, K. E., Wisniewski, T. M., Selkoe, D. J., et al. (2000). Temporal accrual of complement proteins in amyloid plaques in down’s syndrome with Alzheimer’s disease. Am. J. Pathol. 156, 489–499. doi:10.1016/s0002-9440(94)6753-0

Tenner, A. J. (2020). Complement-Mediated events in Alzheimer’s disease: mechanisms and potential therapeutic targets. J. Immunol. 204, 306–315. doi:10.4049/jimmunol.1901068

Tenner, A. J., Stevens, B., and Woodruff, T. M. (2018). New tricks for an ancient system: Physiological and pathological roles of complement in the CNS. Mol. Immunol. 102, 3–13. doi:10.1016/j.molimm.2018.06.264

Wyss-Coray, T., and Rogers, J. (2012). Inflammation in Alzheimer disease–A brief review of the basic science and clinical literature. Cold Spring Harb. Perspect. Med. 2, a006346. doi:10.1101/cshperspect.a006346

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