Acetylcholinesterase-associated inflammation in patients with giant cell arteritis. Evaluation by histology and 11C-donepezil PET/CT

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ABSTRACT

Objective. *To investigate the in-situ expression of acetylcholinesterase (AChE) in the inflamed vessel wall of patients with biopsy-positive giant cell arteritis (GCA) as compared to biopsy-negative non-GCA patients, and to evaluate the in-vivo expression of AChE in patients with large-vessel GCA (LV-GCA) by 11C-donepezil (AChE inhibitor) positron emission tomography/computed tomography (PET/CT).*

Methods. *Twenty-four biopsy-positive GCA and 44 biopsy-negative non-GCA patients were included for AChE histology. Immunohistochemical methods were used to determine the AChE expression. The histological inflammation and the AChE expression were assessed by an experienced pathologist on a 3-point scale. Two patients with newly diagnosed 18F-fluorodeoxyglucose (18F-FDG) PET/CT verified LV-GCA were included for 11C-donepezil PET/CT. PET images were assessed by an experienced nuclear medicine physician.*

Results. *AChE was expressed in all 24 positive temporal artery biopsies, 10/24 showed high AChE expression (grade 2) and 14/24 showed moderate AChE expression (grade 1). No AChE expression was observed outside the media smooth muscle cells (grade 0) in any of the biopsy-negative non-GCA patients. The AChE expression was in 86% agreement with the histological inflammation. The AChE expression was not associated with any clinical or biochemical findings. In both LV-GCA patients, PET/CT revealed extensive vascular FDG uptake but no 11C-donepezil uptake.*

Conclusion. *AChE is highly expressed in the inflamed vessel wall of patients with GCA. Although, 11C-donepezil PET/CT showed no vascular uptake in the FDG PET/CT verified LV-GCA patients, histological findings raise the possibility that AChE can be used in the development of new diagnostic and disease monitoring tools for GCA.*

Introduction

Immune cells have shown the ability to express all the components needed to constitute an independent cholinergic system. These include acetylcholine (ACh), choline acetyltransferase (ChAT), acetylcholinesterase (AChE), and both nicotinic and muscarinic ACh receptors (1-5). Cellular studies have shown that activating T-cells, up-regulates ChAT mRNA expression, ACh synthesis, and AChE activity (6-8). This indicates that immune activation stimulates the cholinergic activity. T-cells have shown a markedly greater ChAT capacity than B-cells, and among T-cells, $CD4^+$ T-cells have shown the highest level and synthetic capacity of ACh (6, 9-11). Since the vascular inflammation in giant cell arteritis (GCA) is dominated by $CD4^+$ T-cells, it is likely that the expression of AChE is increased in the inflamed vessel wall of patients with GCA. A recent study investigating cholinergic positron emission tomography/computed tomography (PET/CT) as a diagnostic tool in infection (12), found very high 11C-donepezil (an AChE inhibitor) uptake in a lobar pneumonia and in peritumoral inflammation surrounding a lung cancer, and moderate uptake in bacterial abscesses. It was suggested that 11C-donepezil could be a more sensitive and specific tracer for certain types of infection/inflammation compared to the 18F-fluorodeoxyglucose (18F-FDG) tracer in PET/CT.

Diagnosing and monitoring disease activity in GCA can be difficult (13).

Neither clinical symptoms, biochemistry, nor imaging tools can stand alone and the final decision often relies on the clinical expert opinion (14). GCA is a chronic medium- and large-vessel vasculitis, affecting cranial arteries (c-GCA) and/or the large vessels (LV-GCA) (15, 16). The temporal artery biopsy (TAB), the traditional gold standard in the diagnosis of c-GCA, has a sensitivity of only 40% (17, 18). Diagnosing LV-GCA often depends on imaging revealing large-vessel inflammation. Due to its high sensitivity, FDG PET/CT is increasingly used to diagnose LV-GCA (19). However, FDG PET/CT also show discrete FDG uptake in atherosclerosis, which may be a clinical challenge in PET centres with low volume of referrals on suspected inflammatory disease (20). Also, FDG PET/CT can remain positive during clinical and biochemical remission and the value of FDG PET/CT for monitoring disease activity in patients with GCA remains to be clarified (21). There is an unmet need for better understanding of the underlying pathological process in the vessel wall leading to FDG PET/CT positivity in GCA and for specific diagnostic and disease activity monitoring tools for GCA. If AChE upregulation can be observed in inflamed tissue in patients with an autoimmune disease such as GCA, it has the potential to be used as a new diagnostic and disease monitoring tool.

To our knowledge the *in-situ* expression of AChE at sites of inflammation has never been investigated. The aim of this study was to investigate the *in-situ* expression of AChE in the inflamed vessel wall of patients with TAB-positive GCA as compared to TAB-negative non-GCA patients, and to evaluate the *in-vivo* expression of AChE in patients with untreated 18f-FDG PET/CT proven LV-GCA by 11C-donepezil PET/CT.

Materials and methods

Participants for AChE histology

Using the electronic pathology biobank register at Department of Histopathology, which keeps records of all TABs performed at Aarhus University Hospital in Denmark, we retrospectively identified 295 patients with suspected GCA who underwent a TAB in the period between January 2012 and December 2015. Clinical data was extracted from electronic medical records and reviewed to verify the diagnosis of GCA. We identified GCA patients with a positive TAB and non-GCA patients with a negative TAB. Non-GCA patients were divided into two groups, patients with polymyalgia rheumatica (PMR) and patients with "other diseases" (*i.e.* infection, osteoarthritis, or cancer). TAB blocks, along with their histological slides, were retrieved from the pathological archive. A pathologist with expertise in vasculitis and blinded to clinical data reviewed the histological slides to confirm the histological diagnosis and to grade the histological inflammation. Only TABs showing moderate to severe inflammation were included. Patients treated with glucocorticoid for >7 days or with any other immunosuppressive were excluded. The process of selection is shown in Figure 1.

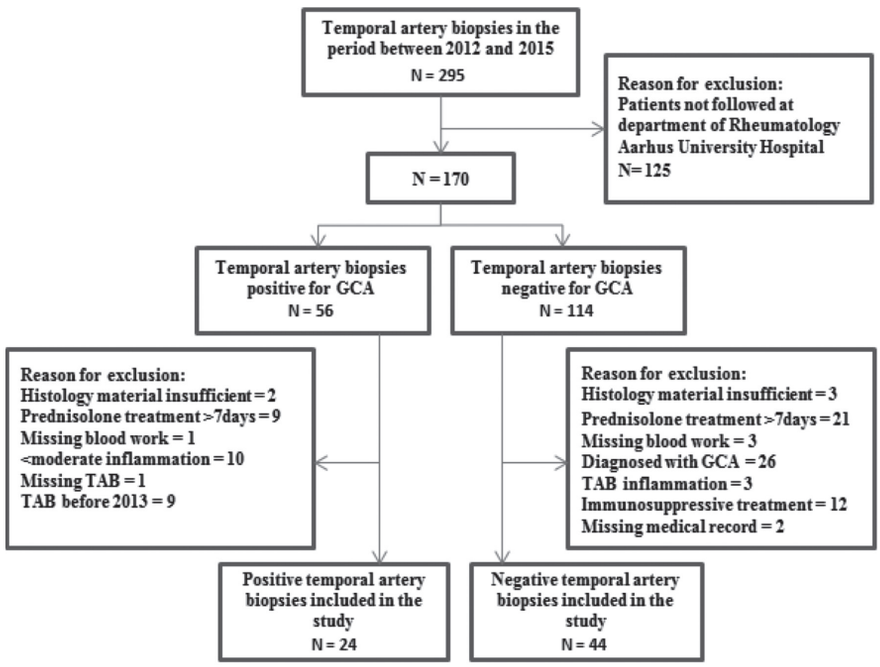

Fig. 1. Selection of the study population. Local ethics comity had approved the inclusion of a total of 25 biopsy-positive giant cell arteritis (GCA) and 50 biopsy-negative non-GCA patients. Due to a sufficient number, biopsy-positive GCA patients before 2013 were excluded.

This study was carried out in accordance with the Helsinki declaration for biomedical research.

The study was approved by the Central Denmark Region Committees on Health Research Ethics [1-10-72-261-15, 1-10-72-60-14] and the Danish Data Protection Agency [1-16-02-663-15, 1-16-02-380-14].

Clinical cases for 11C-donepezil PET/CT

Patients with untreated newly diagnosed LV-GCA at Department of Rheumatology, Aarhus University Hospital were considered for inclusion for 11C-donepezil PET/CT. Patients were eligible if they were >50 years of age, had elevated CRP or ESR, presented with symptoms attributed to GCA, had a FDG PET/CT with aortic and/or supra-aortic large-vessel FDG uptake higher than liver FDG uptake, and did not present with cranial symptoms that required immediate treatment. An extensive evaluation to exclude other potential causes of symptoms and findings was performed by an experienced rheumatologist.

Immunohistochemistry

For immunohistochemical analysis of AChE, new sections of 2.5µm were generated from the formalin fixed and paraffin embedded TABs. Antigens were retrieved using Cell Conditioner 1 (Ventana, Tucson, reference no.950-100, Arizona, USA). Incubation peri-

ods were 2 cycles of 4 minutes at 95°C and 2 cycles of 8 minutes at 100°C. Afterwards, sections were incubated with the primary polyclonal AChE antibody (Nordic Biosite, no. OAAF05614-20UL, Täby, Sweden) in a 1:100 dilution at 37°C for 32 minutes and then incubated with HQ Universal Linker (Ventana, Tucson, reference no. 760-700, Arizona, USA) for 8 minutes. After washing, sections were incubated with OptiView HRP Multimer (Ventana, Tucson, reference no. 760-700, Arizona, USA) for 8 minutes, following incubation with OptiView H_2O_2 and OptiView DAB (Ventana, Tucson, reference no. 760-700, Arizona, USA) for 8 minutes. Lastly, sections were incubated with OptiView copper (Ventana, Tucson, reference no. 760-700, Arizona, USA) for 4 minutes. The staining procedure was performed using a Bencmark XT machine. The optimal concentration of the primary antibody was determined in a series of preliminary experiments. Human cerebral cortex tissue was used as a positive control and staining without the primary antibody was used as a negative control. Additional immunohistochemical staining for CD4, CD8, and CD68 were also performed.

Table I. Characteristics of participants for AChE histology.

	TAB-positive GCA n=24	TAB-negative PMR n=21	TAB-negative "other diseases*" n=23	*p*-value
Demographics				
Age (years)	70.1 (67.5-72.8)	71.6 (67.5-75.6)	68.7 (64.1-73.3)	0.565
Females	16/24 (67)	11/21 (52)	13/23 (57)	0.613
Clinical characteristics				
Patients treated with prednisolone	24/24 (100)	21/21 (100)	8/23 (35)	<0.001*
Time from symptoms to prednisolone treatment (days)	48 (32-73)§	95 (53-170)§	18 (9-36)§	0.002*
Patients treated with prednisolone before biopsy	19/24 (81)	5/21 (24)	8/23 (35)	<0.001*
Cumulative prednisolone dose before biopsy (mg)	204 (136-308)§	136 (81-231)§	197 (73-535)§	0.672
Fever >38 °C	7/23 (30)	4/16 (25)	1/16 (6)	0.211
Weight loss >3kg	11/22 (50)	3/20 (15)	5/17 (29)	0.054
1990 ACR criteria for GCA full-filled#	23/24 (96)	2/21 (10)	7/23 (30)	<0.001*
Headache	20/23 (87)	4/18 (22)	15/21 (71)	<0.001*
New localised headache¤	17/22 (77)	2/18 (11)	9/20 (45)	<0.001*
Jaw claudication	15/23 (65)	3/19 (16)	2/19 (11)	<0.001*
Scalp tenderness	11/16 (69)	2/8 (25)	4/11 (36)	0.093
Unspecific visual symptoms§	14/23 (61)	3/21 (14)	11/21 (52)	0.004*
Permanent loss of vision	3/24 (13)	0/21 (0)	3/21 (14)	0.234
Amaurosis fugax	2/24 (8)	0/21 (0)	1/21 (5)	0.769
Abnormal temporal artery£	14/23 (61)	4/18 (22)	9/19 (47)	0.047*
Inflammatory biomarkers				
B-ESR (mm/hour)	73.9 (62.2-85.5)	39.1 (25.6-52.6)	40.3 (25.0-55.6)	<0.001*
P-CRP (mg/l)	65.8 (44.8-96.5)§	33.3 (20.9-53.0)§	8.0 (3.8-16.6)§	<0.001*

Data are either expressed as mean (95% CI), geometric mean (95% CI)§, or as no. (%)
*Cancer, osteoarthritis, infection, or ischaemic vascular disease.
#Number of patients who fulfilled the 1990 ACR criteria for GCA.
¤New uni- or bilateral headache localised in either the occipital, frontal, or the temporal region.
§Includes symptoms of blurred vision, decreased eyesight, and/or flicker of the eyes.
£Tenderness, thickening, and/or decreased pulse of the temporal artery.

Western blotting

Western blotting was performed to validate the AChE antibody. It was carried out using human and rat cerebral cortex total protein lysate. The rat cerebral protein was produced at our lab and the human cerebral protein was purchased at Abcam (Abcam, no. ab30061, Cambridge, UK). Samples of 10µg protein were loaded into a criterion TGX StainFree 4-15% gel (BioRad, no. 5678085, California, USA) and run at 160V for 85min. Proteins were subsequently transferred to a membrane. The membrane was placed in the primary polyclonal AChE antibody (Nordic Biosite, no. OAAF05614-20UL, Täby, Sweden) in a 1:500 dilution at 4°C overnight. The membrane was subsequently placed in the secondary antibody (Goat anti-rabbit IgG-HRP, Santa Cruz, no. sc-2054, Texas, USA) in a 1:10000 dilution for 90min at room temperature. Blots were developed using BioRad Clarity.*Histological evaluation*

The histological inflammation on HE-slides and the expression of AChE were evaluated by a pathologist with expertise in vasculitis, blinded to clinical data. Both the histological inflammation and the AChE expression were graded on a semi-quantitative 3-point scale (0–2). AChE staining in the layer of smooth muscle cells was not included in the assessment.

11C-donepezil PET/CT

Patients refrained from any intake of calories at least 6 hours before the scan. Patients were injected with 400 MBq of 11C-donepezil and 20 minutes after injection, a low-dose CT and PET scan was performed of the neck, thorax, and abdomen (22).

PET images were assessed by an experienced nuclear medicine physician. Previous 11C-donepezil PET studies have shown that discernible tracer uptake is almost never seen in the wall of large arteries. Therefore, 11C-donepezil uptake in large arteries of GCA patients was visually assessed as present or absent and compared to baseline FDG uptake in large arteries.

Statistics

Statistical analysis was performed using StataCorp. 2015. Stata Statistical Software: Release 14 (College Station, TX: StataCorp LP).

Baseline characteristics of participants for AChE histology were compared using one-way ANOVA or Fisher's exact test. The assumption of normality was assessed using QQ-plots and variance homogeneity was assessed using Bartlett's test. A significance level of $p<0.05$ was deemed statistically significant. The agreement between the AChE expression and the histological inflammation was determined using Cohen's kappa.

Results

Baseline characteristics of the participants for AChE histology

A total of 24 TAB-positive GCA and 44 TAB-negative non-GCA patients were included (Fig. 1). Of the 44 TAB-negative non-GCA patients 21 were diagnosed with PMR and 23 with other diseases. Baseline characteristics of study participants are shown in Table I. Histological characteristics of included TABs are shown in Table II.

Expression of AChE

AChE was expressed in all positive TABs. The expression of AChE is shown in Table III. AChE was expressed in areas with $CD4^+$ T-cells, $CD8^+$ T-cells, and $CD68^+$ cells (macrophages). Areas without leukocyte infiltration, not including the media smooth muscle cells (SMCs), showed no expression of AChE (Fig. 2). Negative TABs from non-GCA patients showed no expression of AChE outside media SMCs (*i.e.* grade 0) (Fig. 2). $CD4^+$ and $CD8^+$ T-cells were present in all the positive TABs; none of the negative TABs showed any significant presence of $CD4^+$ and $CD8^+$ T-cells. $CD4^+$ T-cells were more abundantly present than $CD8^+$ T-cells (Fig. 2). Cross tabulation of the graded AChE expression and histological inflammation showed an 86% agreement, with a $p<0.001$ and a Cohen's kappa value of 0.79.

We compared clinical features (*i.e.* classic cranial symptoms, constitutional symptoms, and biochemistry) of GCA patients with moderate AChE expression (n=14) to GCA patients with high AChE expression (n=10). No symptoms, clinical findings, or biochemistry showed any significant differences.

Validation of AChE antibody

Western blot showed bands around 55, 58, and 71 kDa (Fig. 2). Similar bands have been reported in other studies (11, 23, 24). IHC of cerebral cortex showed specific staining of neurons as expected (Fig. 2). Neither the WB nor the IHC showed any bands/staining when the primary antibody was omitted from the staining procedure.

Table II. Histological characteristics.

	Positive (n=24)	Negative (n=44)	*p*-value
Severe transmural inflammation	11/24 (46)	0/44 (0)	<0.001*
Moderate transmural inflammation	13/24 (54)	0/44 (0)	<0.001*
Giant-cells	17/24 (71)	0/44 (0)	<0.001*
Intimal hyperplasia*	23/24 (96)	27/44 (61)	0.002*
Fragmentation of the internal elastic lamina[a]	23/24 (96)	13/44 (30)	<0.001*
Calcification	1/24 (4)	6/44 (14)	0.407

Data are expressed as no. (%).
*Defined as tunica intima thickness ≥ that of tunica media.
[a]Defined as fragmentation of ≥25% of the circumference of the internal elastic lamina.

Table III. Expression of AChE.

	AChE expression		
	Grade 0	Grade 1	Grade 2
TAB-positive GCA	0/24	14/24	10/24
TAB-negative non-GCA	44/44	0/44	0/44

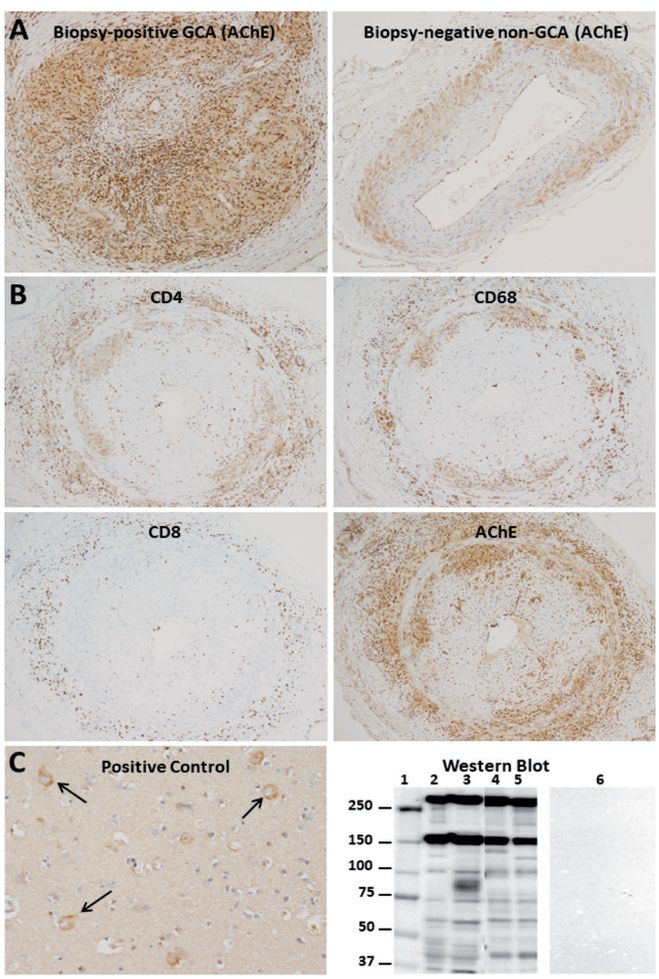

Fig. 2. Expression of acetylcholinesterase (AChE).
A: AChE staining of a positive and negative temporal artery biopsy (TAB). The negative TAB show intimal hyperplasia and AChE staining in the smooth muscle cells.
B: AChE, CD4, CD8, and CD68 staining of a positive TAB. Areas with CD4+, CD8+, and CD68+ cells show high AChE expression; areas without leukocyte infiltration show no AChE expression.
C: Positive Control - AChE staining of human cerebral cortex, arrows show AChE positive neurons. AChE Western blot of human/rat cerebral cortex total protein, 1: Marker, 2: Human, boiled, 3: Human, unboiled, 4: Rat, boiled, 5: Rat, unboiled, 6: Blank.

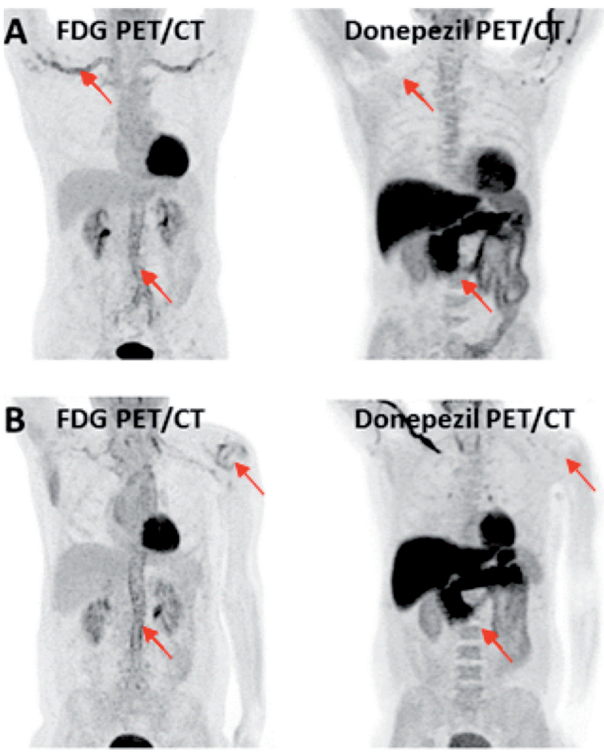

Fig. 3. 11C-donepezil PET/CT.
A: FDG PET/CT and 11C-donepezil PET/CT of the older large-vessel giant cell arteritis (LV-GCA) patient. FDG PET/CT shows high intensity FDG uptake in the abdominal aorta, the subclavian, and in the axillary artery. No vascular uptake is seen on the 11C-donepezil PET/CT.
B: FDG PET/CT and 11C-donepezil PET/CT of the younger LV-GCA patient. The FDG PET/CT shows high intensity FDG uptake in the abdominal aorta, at the greater trochanter, in the spinous process of the cervical and lumbar vertebra, and in the periarticular area of the left scapulohumeral joint. No vascular uptake is seen on the 11C-donepezil PET/CT.

Baseline characteristics of LV-GCA patient for 11C donepezil PET/CT

Two untreated male LV-GCA patients, aged 57 and 75 years, were included for an 11C-donepezil PET/CT (Fig. 3). Both patients reported muscle pain, morning stiffness for >½ hour, fatigue, intermittent fever >38°C, weight loss (10 and 7 kilograms), and profuse night sweats. The youngest patient had classical PMR symptoms with pain, stiffness, and limited range of motion in the neck, shoulders, lower back, and hips, while the older patient experienced uncharacteristic muscle pain in the chest and in the knees. Laboratory test showed CRP 9.9 and 52.0 mg/L, and ESR 20 and 87 mm/h, respectively. A FDG PET/CT was performed in both patients showing high intensity FDG uptake consistent with LV-GCA and PMR (Fig. 3).

A TAB was performed in both patients after 13 days of treatment. TAB from the youngest patient showed focal lymphoplasmacytic inflammation accompanied by macrophages with incipient giant cell formation; in the older patient TAB revealed lymphoplasmacytic inflammation in the adventitia. Both TABs showed intimal hyperplasia and fragmentation of the internal elastic lamina.

11C-donepezil PET/CT

11C-donepezil PET/CT was performed before glucocorticoid treatment was initiated in both patients. In none of the patients, 11C-donepezil uptake was present in any of the large arteries (Fig. 3). As an additional observation, 11C-donepezil uptake was also not detected in any of the bursae or periarticular areas showing high intensity FDG uptake considered consistent with PMR (Fig. 3).

Discussion

This is the first evaluation of the *in-situ* expression of AChE at sites of inflammation. We found that AChE was expressed in all inflamed TABs and that the AChE expression was in good agreement with the histological inflammation. No expression of AChE was observed outside the media smooth muscle cells (SMCs) in non-inflamed TABs from non-GCA patients. SMCs express cholinergic receptors and are innervated by the parasympathetic nervous system, which uses ACh as its main neurotransmitter (25). Therefore, we expected SMCs to show some expression of AChE in all TABs. The AChE expression in inflamed TABs was confined to areas with $CD4^+$ and $CD8^+$ lymphocytes, and $CD68^+$ macrophages. Apart from the media SMCs, areas with no immune cell infiltration, showed no expression of AChE. This supports the assumptions that activated immune cells are the main source of AChE in the inflamed TABs. This is consistent with immune cells up-regulating cholinergic components when activated (6-8). $CD4^+$ and $CD68^+$ cells were more abundantly present than $CD8^+$ cells in the inflamed TABs. This is consistent with GCA being a $CD4^+$ T-cell and macrophage dominated disease. Among immune cells, $CD4^+$ T-cells have shown the highest level and synthetic capacity of ACh, which is why we expected the $CD4^+$ T-cell dominated inflammation in GCA to increase the expression of AChE (6, 9-11). The expression of AChE in inflamed but not in non-inflamed TABs indicates that AChE could play a significant role in the inflammatory process in GCA.

Surprisingly, the AChE inhibitor 11C-donepezil showed no uptake in any of the large arteries of the two treatment-naïve, LV-GCA patients included in this study. FDG PET/CT of both patients had shown extensive vascular uptake 3 days prior to the 11C-donepezil PET/CT. No glucocorticoid treatment had been initiated in between the two scans. Based on the clear AChE histology results we expected the 11C-donepezil PET/CT to show vascular uptake in the two patients with active LV-GCA. Previous PET/CT studies have shown very high 11C-donepezil uptake in a bacterial pneumonia and in the peritumoral inflammation surrounding a non-small cell lung carcinoma (12). Immune responses differ between acute bacterial infection, peritumoral inflammation, and the autoimmune inflammation in GCA, which may result in 11C-donepezil uptake in infectious disease but not in GCA. Also, the density of AChE in the inflamed vessel walls may be insufficient to provide a discernible 11C-donepezil PET/CT signal due to the limited spatial resolution of the PET.

Human arteries show heterogeneity in their immune expression (26). Since arteries show region specific immune expression, it could explain why AChE is seen in the inflamed TABs using histology, but not in the large thoracic and

abdominal vessels using 11C-donepezil PET/CT. Both LV-GCA patients included in this study had positive TABs. We did not include the head in the PET scan, so it is possible that 11C-donepezil signal may have been visible in the temporal arteries of these patients.

We found no significant clinical differences between GCA patients with high and moderate AChE expression. This indicates that the AChE expression at the level of the TAB does not measure GCA disease severity. However, the study population (n=24) is relatively small and the study design is not optimal for such an evaluation. Therefore, these results should be interpreted with caution.

By design, our study has both strengths and limitations. The retrospective design of the histology study is one of its weaknesses. We assessed medical records to verify patient diagnosis, but clinical data according to the 1990 ACR classification criteria for GCA were missing in some cases. This increases the risk of misclassification. However, we only included GCA patients with TABs showing moderate to severe inflammation and we excluded TAB-negative GCA patients. This ensured a valid GCA diagnosis and 96% of GCA patients fulfilled the 1990 ACR classifications criteria for GCA and were treated as such. Only very few studies have investigated the expression of AChE using immunohistochemical methods on formalin fixed, paraffin embedded tissue (27). We validated the IHC method using both human cerebral cortex as a positive control and performed Western blotting on cerebral cortex protein.

In conclusion, our study shows that AChE is highly expressed in inflamed TABs from patients with GCA. Although, 11C-donepezil PET/CT showed no vascular uptake in the two FDG PET/CT verified LV-GCA patients, the histological findings raise the possibility that AChE can be used in the development of new diagnostic and disease monitoring tools for GCA.

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