

SHORT REPORT

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# Expression and processing analyses of wild type and p.R47H TREM2 variant in Alzheimer's disease brains

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## Abstract

**Background:** Genetic analyses showed that the triggering receptor expressed in myeloid cells 2 (TREM2) p.R47H variant increases the risk for Alzheimer's disease (AD). The question of whether the p.R47H mutation affects expression or function of the receptor remains unanswered. To address this question we quantified mRNA and analyzed protein profiles of WT and p.R47H TREM2 in human brains.

**Methods:** Quantitative real-time PCR (qPCR) was performed using 2 sets of primers one that detects all *TREM2* mRNA isoforms and one specific for the alternative spliced isoform (*TREM2alt*) that encodes for the extracellular domain (soluble TREM2). Because in the brain TREM2 is expressed primarily in microglial cells, we also assessed the levels of *IBA1* to control for microglial variability across samples. For TREM2 protein quantitation and N-glycosylation processing, RIPA brain extracts were analyzed by Western blot before and after EndoH and PNGaseF treatments.

**Results:** We identified statistically significant increased levels of *TREM2* transcripts in the temporal cortex of AD subjects when compared with controls; *TREM2alt* was likewise higher in AD cases, but was not significant after adjustment for covariates. Quantitative analysis of TREM2 protein confirmed qPCR results that showed higher levels in AD than in control brains. Among AD subjects, we observed a trend towards higher mRNA and protein TREM2 levels in carriers of the p.R47H risk allele. Analysis of individual TREM2 species found no difference in the relative amounts of mature and immature species, and carboxyl terminal fragments between non carriers and p.R47H samples. Furthermore, TREM2 species from either non carriers or p.R47H brains were equally susceptible to EndoH and PNGaseF treatments.

**Conclusions:** Our results suggest that TREM2 expression is increased in AD. Furthermore, we provide evidence indicating that p.R47H mutation does not affect the levels of TREM2 either directly by altering expression or indirectly by affecting processing of the protein. Our data support previous findings that suggest that p.R47H variant affects TREM2 function by altering binding properties of the receptor rather than expression.

**Keywords:** AD, TREM2, R47H, Microglia

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## Introduction

Alzheimer's disease (AD) is the leading cause of dementia in the elderly. AD pathology is characterized by extracellular plaques composed primarily of amyloid beta protein (A $\beta$ ) and intracellular neurofibrillary tangles composed primarily of hyperphosphorylated tau. According to the amyloid cascade hypothesis, neurofibrillary tangles and the neuroinflammation invariably observed in AD are downstream events caused by A $\beta$  accumulation [1]. There is mounting evidence, however, that the innate immune system plays an important role in AD. Support for involvement of innate immunity came from genetic and integrated system studies as follows. i) Network-based integrative analysis identified an immune/microglia module as one of the molecular systems most strongly associated with the pathophysiology of late onset AD [2]. ii) Genome-wide association studies identified genes of the immune system in general (*CLU* and *MS4A*) and of microglia function in particular (*CD33* and *CR1*), in or close to the risk loci for late onset AD [3, 4]. iii) Heterozygous variants in triggering receptor expressed in myeloid cells 2 (*TREM2*), which encodes for a microglia receptor, associate with increased risk for AD. [5, 6]. Among these rare variants, p.R47H is the most commonly found associated with AD.

*TREM2* belongs to the *TREM* family of receptors whose members are cell surface glycoproteins possessing an immunoglobulin-like extracellular domain, a transmembrane region, and a short cytoplasmic tail. In the brain, *TREM2* is involved in regulation of the microglia inflammatory response and phagocytosis of cell debris. The receptor relies entirely on the adaptor protein DAP12 (aka *TYROBP*) for intracellular signaling, and this partnership is absolutely required for efficient phagocytosis [7].

Loss of function for either *TREM2* or DAP12 has clinical implications, resulting in polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOS), a rare and fatal disease also known as Nasu Hakola disease, characterized by bone cysts and late-onset dementia [8, 9]. Although, the mechanism behind the pathology in PLOS is not known, one hypothesis is that lack of either *TREM2* or DAP12 activity impairs the clearance of apoptotic neurons by microglia, leading to the accumulation of necrotic debris [10].

It was recently demonstrated that *TREM2* undergoes a proteolytic cleavage that releases its extracellular domain, leaving a carboxy terminal fragment (CFT) attached to the membrane [11]. In addition to the membrane-bound form, a soluble form of *TREM2* (s*TREM2*) is detectable in plasma and CSF [12]. It is assumed that s*TREM2* originates by the release of the receptor extracellular domain; however, in human brains the presence of an alternatively

spliced *TREM2* transcript (*TREM2 alt*) encoding for s*TREM2* has been reported [13].

In this study we used autopsy brains to quantitate *TREM2* and *TREM2alt* transcript levels in two cohorts: one composed of AD and normal control subjects and the other of p.R47H carriers and non-carriers (wild type). We also analyzed *TREM2* at the protein level in p.R47H carriers, and compare its N-glycosylation profile with wild type brains.

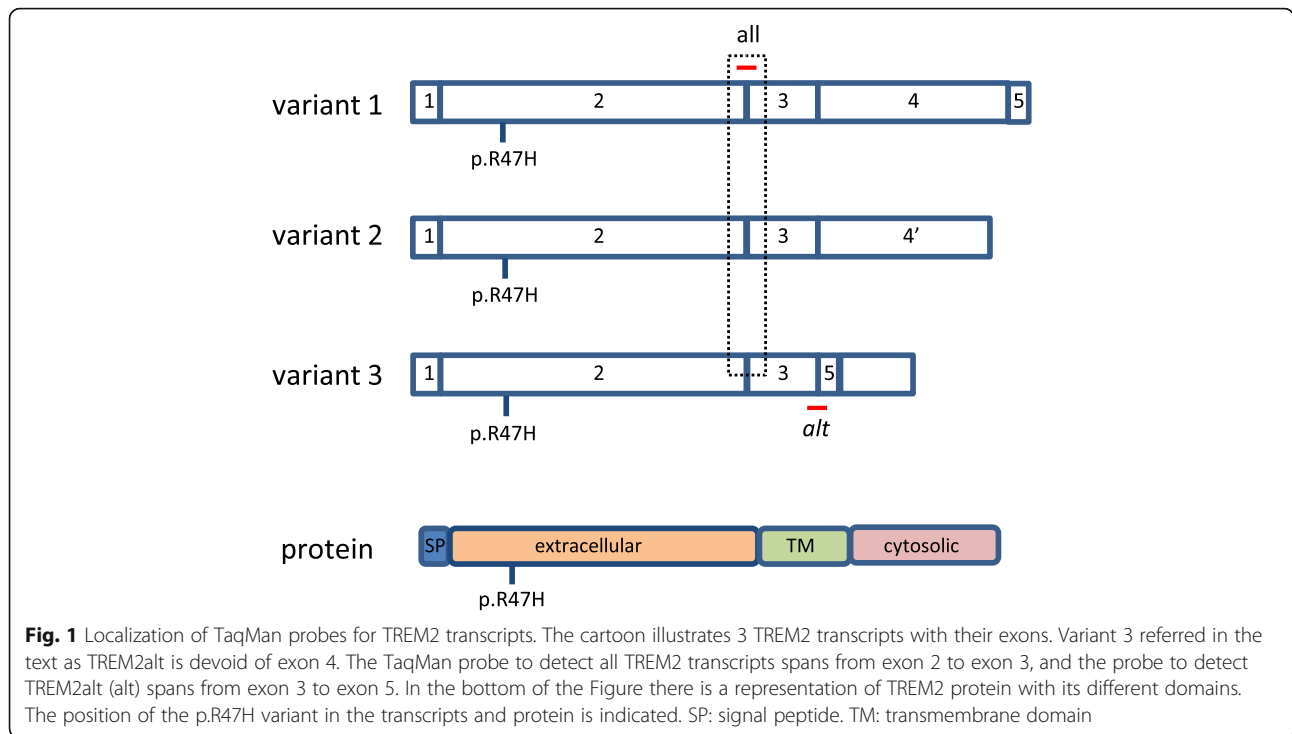
## Findings

### Quantitative analysis of *TREM2* transcripts in control and AD brains

There are at least three *TREM2* transcripts that are expressed in human brain [13]. Variant 1, the longest *TREM2* transcript consists of 5 exons, while variant 3 (the shortest) is an alternatively spliced isoform that excludes exon 4 (Fig. 1). Exon 4 contains the sequence for the *TREM2* transmembrane domain; thus, variant 3 that was recently detected in human brains [13], likely encodes for a soluble form of the receptor (s*TREM2*). Previous studies using transfected cells suggest that proteolytic cleavage of the extracellular domain is the main mechanism by which s*TREM2* is generated [11, 14]. Regardless of the mechanism, the potential relevance of s*TREM2* as biomarker to monitor AD progression is highlighted by its elevation in the CSF of AD patients [15, 16].

In principle, the elevation of s*TREM2* in AD patients could be the result of an overall increase of *TREM2* expression and/or a specific increase in s*TREM2* production. To address these possibilities, we quantified levels of total *TREM2* transcripts and the alternatively spliced variant 3 (*TREM2alt*) in the temporal cortex of normal control and AD from post-mortem brains.

Demographics of the control and AD cases (cohort A) used for the analysis are summarized in Table 1. RNA was reverse transcribed and qPCR was performed using two sets of TaqMan primers: one that detects all *TREM2* mRNA variants and one specific for *TREM2alt*. Based on  $\Delta C_t$  calculations, the level of *TREM2alt* was between 5 and 7 times lower than of all *TREM2* transcripts combined. Relative expression levels were determined for all comparisons using the  $\Delta\Delta C_t$  method ( $2^{-\Delta\Delta C_t}$ ) and tested for differences between groups using the Wilcoxon rank sum test. We found statistically significant increased levels of *TREM2* transcripts in the temporal cortex of AD subjects when compared to controls ( $p = 1.14E-05$ , Table 2 and Fig. 2a). *TREM2alt* was also increased in AD ( $p = 7.28E-05$ , Table 2 and Fig. 2a) and the expression levels of *TREM2* and *TREM2alt* were correlated ( $r > 0.72$ , data not shown). The extent of the increase in AD brains was similar for both total *TREM2* and *TREM2alt* suggesting that the increase is not likely to be



transcript-specific. To further assess significant results identified by the non-parametric Wilcoxon rank sum test (Table 2), we performed multivariable linear regression, using  $\Delta Ct$  as the expression variable, adjusting for known technical and biological covariates, and levels of the microglial marker *AIF1* (aka *IBA1*). Since we used  $\Delta Ct$  as the expression variable in this analysis, it should be noted that a negative beta indicates increased expression (Table 2). We confirmed the association of increased *TREM2* levels in the temporal cortex of AD compared to controls ( $p = 2.30E-02$ , Table 2) and although the increase for *TREM2alt* was not significant ( $p = 1.41E-01$ , Table 2), the association was in the same direction. This result indicates that *TREM2* levels are increased in AD even after taking into account the expression of the microglial marker *IBA1*; therefore, it is unlikely that the observed increase in *TREM2* expression is entirely due to increase number of microglial cells. Furthermore, we observed a positive correlation

between *TREM2* and *TREM2alt* with *IBA1* expression levels in control and AD samples (Fig. 2c).

*TREM2* expression analysis in cerebellum (a brain region with minimal neuronal loss in AD) was confounded by the low levels of *TREM2* in that brain region; however, the same trend towards increased expression in AD compared to controls was observed (data not shown).

Similar analysis was performed with RNA from an independent cohort (B) of p.R47H *TREM2* carriers (CT) and those homozygous for the major allele (CC) (referred also in the text and Figures as wild type). All p.R47H carriers had pathologically-confirmed typical AD, except for one sample that had posterior cortical atrophy (PCA), an AD subtype; wild type (WT) non carriers used for the analysis were also AD and one was PCA. The Wilcoxon-rank-sum test indicated higher *TREM2* and *TREM2alt* levels in carriers of the p.R47H

**Table 1** Demographic of studied cases

	N	Mean age at death (SD)	Female: N (%)	ApoE4: N (%)	RIN: median (range)
AD <sup>a</sup>	33	72.7 (6.2)	17 (52%)	16 (48%)	6.0 (5.0–7.9)
CONT <sup>a</sup>	33	70.7 (5.7)	18 (55%)	15 (45%)	7.0 (5.3–8.4)
WT (CC) <sup>b</sup>	26	79.7 (8.5)	19 (73%)	20 (77%)	6.6 (5.5–8.6)
R47H (CT) <sup>b</sup>	15	81.5 (8.3)	11 (73%)	9 (69%)	6.3 (5.5–8.4)

<sup>a</sup>cohort A <sup>b</sup>cohort B

**Table 2** Summary of expression association analysis

Dx	Transcript	Wilcoxon rank sum ( $2^{-\Delta\Delta Ct}$ )		Linear Regression ( $\Delta Ct$ )	
		Median (IQR)	p-value	Beta (se)	p-value
AD <sup>a</sup>	<i>TREM2</i>	3.19 (2.03–4.06)	1.14E-05	-0.51 (0.22)	2.30E-02
AD <sup>a</sup>	<i>TREM2alt</i>	2.62 (2.10–3.75)	7.28E-05	-0.33 (0.22)	1.41E-01
AD (R47H) <sup>b</sup>	<i>TREM2</i>	1.20 (1.04–1.74)	7.61E-02		
AD (R47H) <sup>b</sup>	<i>TREM2alt</i>	0.97 (0.75–1.44)	6.02E-01		

<sup>a</sup>cohort A <sup>b</sup>cohort B

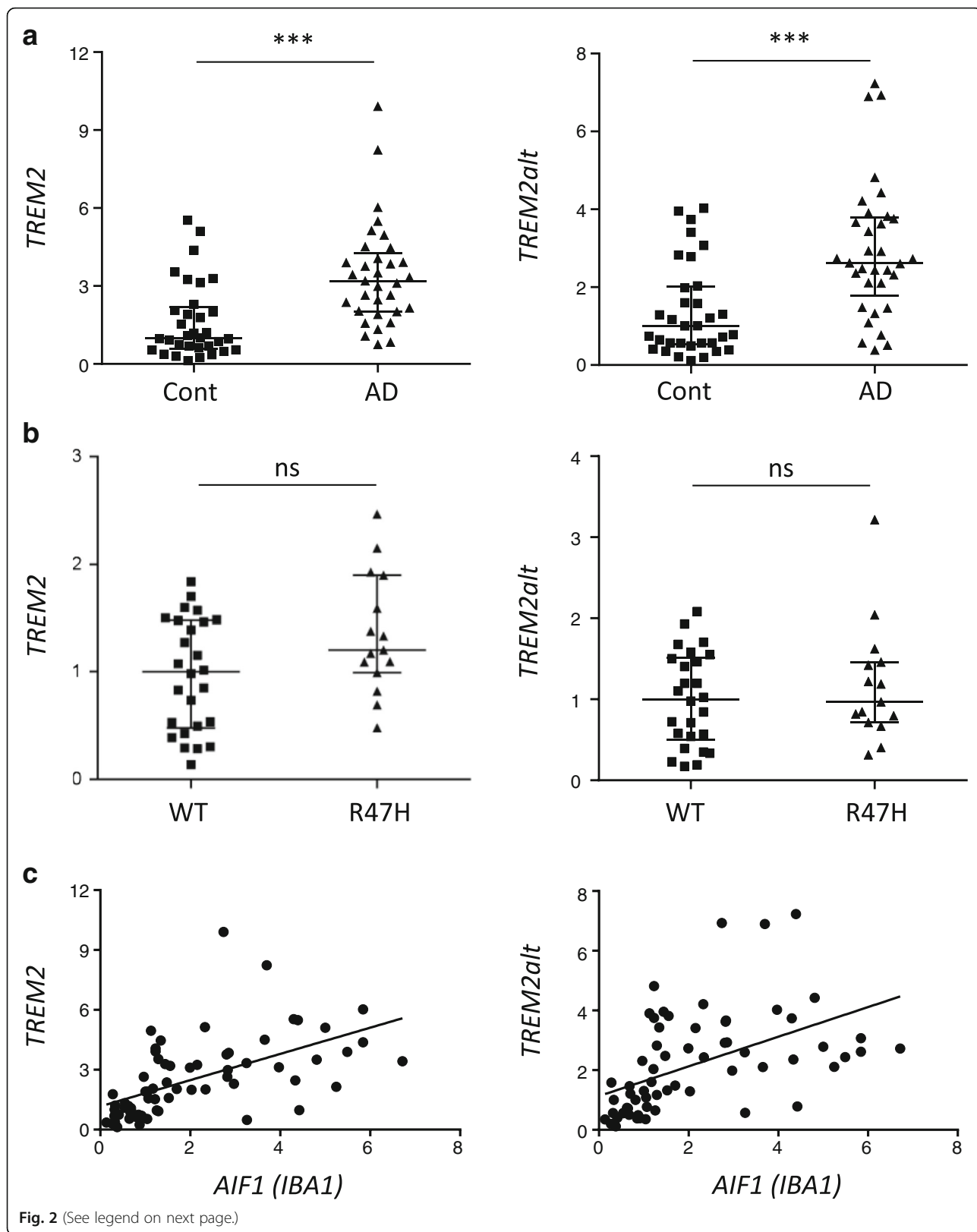


Fig. 2 (See legend on next page.)

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**Fig. 2** Expression of total and alternative spliced *TREM2* mRNA in AD and p.R47H carriers. Relative quantitation was performed using  $2^{-\Delta\Delta C_t}$  (fold change) method. For each sample in the AD or p.R47H group the fold change (FC) value is expressed relative to the median value of *TREM2* in control (a) or WT group (b) that was set at 1. The median FC value in AD samples is 3.19 for *TREM2* and 2.62 for *TREM2alt*; and in p.47H carriers is for *TREM2* 1.20 and 0.97 for *TREM2alt*. \*\*\* $p < 0.0001$ . ns: not significant. c. Assessment of relative expression levels ( $2^{-\Delta\Delta C_t}$ ) for *TREM2* or *TREM2alt* indicates a positive association in all the samples (controls and ADs) for both *TREM2* transcripts with the microglial marker *AIF1* (*IBA1*) (R square values of 0.32 and 0.25 for *TREM2* and *TREM2alt*, respectively; and  $p < 0.0001$  for both transcripts)

risk allele; however, differences between the two groups were not statistically significant (Fig. 2b and Table 2).

### Quantitative analysis of *TREM2* protein in control and AD brains

Protein analysis was performed in the same brain samples in which *TREM2* transcripts were previously evaluated. We performed quantitative Western blot to establish if the increase in *TREM2* mRNA in AD samples is reflected at the protein level. RIPA buffer extracts from temporal cortexes from a subset of control and AD samples ( $N = 48$  out of 66) analyzed for *TREM2* transcripts were used and the blots were probed with a monoclonal antibody against the carboxy terminus of *TREM2*. This antibody allowed the detection of three *TREM2* species: mature (~50 kDa), immature (~30 kDa), and carboxy terminal fragment (CTF) (Fig. 3a). The CTF species were identified on Western blots based on its molecular weight and immunoreactivity with a C-terminal antibody (Fig. 3a), but not with a N-terminal antibody (data not shown). The designation as mature or immature *TREM2* species was based on their resistance or susceptibility to EndoH and PNGaseF, respectively (Fig. 4c). Quantitation of all *TREM2* species normalized to *Iba1* reveals a statistically significant elevation in AD samples as compared to controls (Fig. 3b). To evaluate *TREM2* processing we also compared the ratio between immature and mature species and the levels of CTF species between the two groups. The ratio between immature and mature *TREM2* was significantly higher ( $p < 0.001$ ) in the AD samples, but not significant differences were detected when the levels of CTF species between controls and ADs were compared (Fig. 3b).

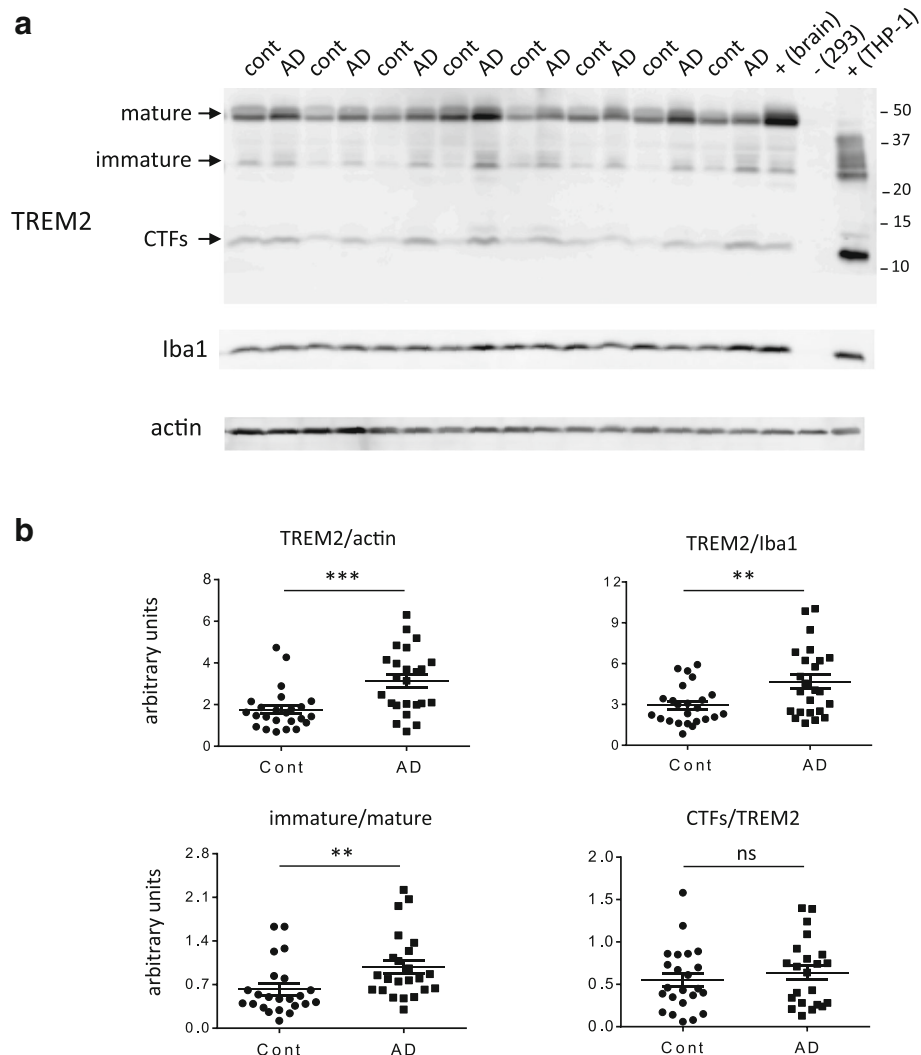
As might be expected given the observed increase of both *TREM2* RNA and protein in AD subjects when compared with controls, Spearman rank analysis indicated a positive correlation between *TREM2* mRNA and protein values ( $\rho = 0.36$ ,  $p = 0.012$ ) when assessed in the same subjects. Our data are in agreement with a previous study of postmortem human brains that reported higher *TREM2* protein levels in AD than in controls [17].

### Expression and deglycosylation profiles of WT and p.R47H *TREM2*

To establish if the p.R47H mutation alters either the expression and/or glycosylation of *TREM2*, we subjected

brain samples from WT and p.R47H carriers to quantitative Western blot analysis and enzymatic treatments with glycosidases. The distribution of *TREM2* signal between mature, immature, and CTF species varied among samples (Fig. 4a), but statistical analysis shows no significant difference in total *TREM2* expression between WT and p.R47H carriers (Fig. 4b). Furthermore, the ratio between immature and mature species is not altered by the presence of the mutation, suggesting that it does not affect intracellular trafficking of the receptor (Fig. 4b). We also looked at the impact of p.R47H on the extent of *TREM2* proteolytic cleavage by quantitating CTF species relative to total *TREM2* species in each sample and found no difference with WT subjects (Fig. 4b). This data indicates that the p.R47H mutation does not affect *TREM2* delivery to the cell surface where CTF species are generated. The intracellular trafficking of *TREM2* can be monitored by assessing modifications to its N-glycan chains using EndoH and PNGaseF enzymes. EndoH susceptible *TREM2* species (immature) are still in the endoplasmic reticulum while resistant (mature) have left that compartment. All N-glycosylated species are susceptible to PNGaseF regardless of their intracellular localization. Removal of N-glycan chains from *TREM2* by either EndoH or PNGaseF results in a lower molecular weight of the protein, a change that can be detected by Western blot. Figure 4c shows representative deglycosylation experiments of WT and p.R47H samples using EndoH and PNGaseF enzymes with different proportions of ~50 kDa and ~30 kDa *TREM2* species. We consistently observed that in WT and p.R47H samples the ~50 kDa species (mature) was resistant to EndoH and susceptible to PNGaseF and that the ~30 kDa species (immature) was susceptible to both enzymes. The nature of the molecular weight difference between mature and immature *TREM2* species is unknown; however based on the enzymatic deglycosylation experiments the molecular weight difference is not due to glycosylation (Fig. 4c).

Our results contrast with a recent study that found elevated mRNA but decreased protein expression in p.R47H carriers [18]. Our data indicate a trend, albeit not significant, towards both higher mRNA and protein *TREM2* levels as a result of the mutation. Furthermore, our results do not support previous in vitro studies that suggested that p.R47H affects *TREM2* intracellular



**Fig. 3** TREM2 protein expression in control and AD brains. Temporal cortices were extracted with RIPA buffer and 100 µg of the soluble fraction (supernatant of 20,000 g centrifugation for 30 min) was used for the analyses. **a**. Western blot of a representative gel probed with a C-terminal TREM2 antibody, *Iba1*, and actin antibodies. Three main TREM2 species are indicated: mature, immature and carboxy terminal fragment (CTF). To confirm the specificity of the C-terminal TREM2 antibody two positive and one negative controls were included. The positive (+) controls were lysates of a human brain and THP-1 cells (a human monocyte cell line) previously tested with B-3, a well characterized TREM2 antibody. The negative (-) control was a lysate of 293 cells that do not express endogenous TREM2. **b**. The signal intensity of all three species TREM2 species, *Iba1*, and actin in each sample was quantitated using ImageQuant software and used for plotting TREM2 (all species) normalized to actin and *Iba1* levels, the ratio between immature and mature species, and CTF species normalized to TREM2 (full length: immature and mature species). \*\*\* $p < 0.0001$ , \*\* $p < 0.001$ , and ns: not statistically significant

trafficking [14, 19]. Rather, our data are more in line with a recent study showing higher TREM2 levels in CSF of p.R47H carriers than in controls [12].

In conclusion, we demonstrated that total *TREM2* including *TREM2alt* transcript and TREM2 protein levels are increased in AD. Our study indicates that proteolytic cleavage of the receptor is likely the main mechanism for sTREM2 generation in the brain based on the very low levels of expression of *TREM2alt* and the detection of CTF species in brain extracts. Finally, our data supports the notion that the p.R47H mutation does not

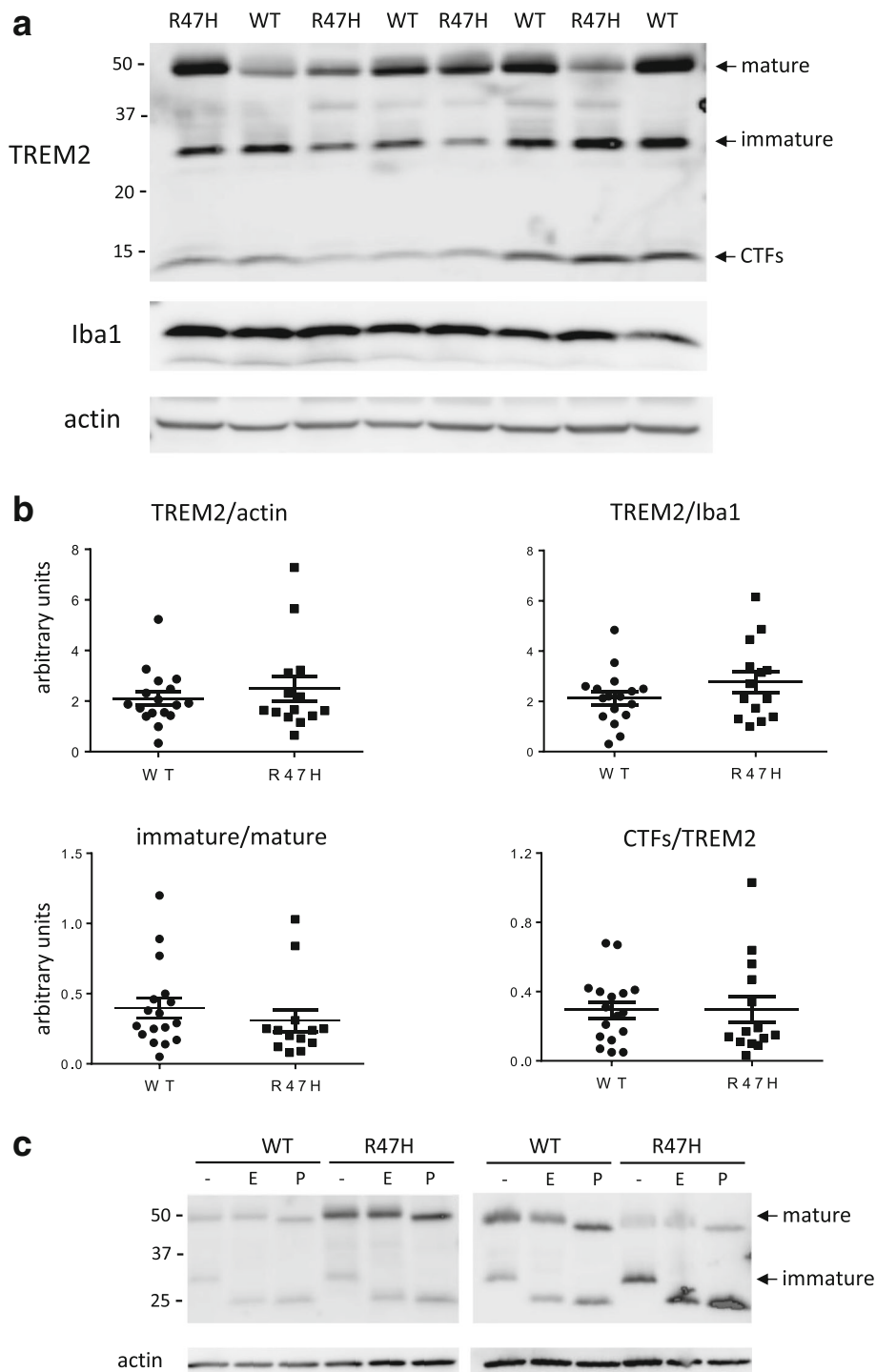
affect processing (i.e., N-glycosylation and cell surface proteolytic cleavage) of TREM2.

## Material and methods

### Brains

In the autopsy-confirmed series, all brains were from the brain bank at Mayo Clinic in Jacksonville. The diagnosis of confirmed AD was made according to accepted neuropathological criteria. All late-onset AD brains analyzed had a Braak neurofibrillary tangle stage of IV or greater. Brains employed as controls had a Braak stage





**Fig. 4** TREM2 expression and deglycosylation profiles in WT and p.R47H carriers. RIPA extracts from temporal cortices were used for the analyses. **a.** Western blot of a representative gel probed with C-terminal TREM2 antibody, Iba1, and actin antibodies. Three main TREM2 species are indicated: mature, immature and carboxy terminal fragment (CTF). **b.** The signal intensity in TREM2 species in WT and p.R47H samples ( $N = 16$  for each group) was quantitated as previously described, and was plotted after normalization to actin and Iba1. The ratio between immature and mature TREM2 species and between CTF species normalized to TREM2 (full length: immature and mature species) were also plotted. **c.** Soluble fractions from RIPA buffer brain extracts were incubated in reaction buffers without enzymes (controls) or with endoH (1000U) or PNGaseF (1000U) overnight at 37 °C. The whole reaction mixture was analyzed by Western blot probed with a C-terminal TREM2 antibody. Blots containing two representative samples from WT and p.R47H brains are shown

of less than III. Most had other brain pathology unrelated to AD and included cerebrovascular pathology, frontotemporal dementia, Lewy body diseases, corticobasal degeneration, argyrophilic grain disease, multi-system atrophy, amyotrophic lateral sclerosis, and progressive supranuclear palsy. No subjects in this study carried familial Alzheimer's disease mutations in APP, PSEN or the TREM2 R47H mutation, unless indicated.

#### qPCR

Total RNA was extracted from 50 mg of frozen temporal cortex and cerebellum of late-onset Alzheimer's disease brains and controls with Trizol (Sigma). Pure-link DNase (Invitrogen) was used to remove genomic DNA, and RNA was reverse transcribed to single-stranded cDNA using the High-Capacity cDNA Archive Kit from AppliedBiosystems. Quantitative real-time PCR was performed in triplicate for each sample using ABI TaqMan Low Density expression Arrays (384-Well Micro Fluidic Cards) with pre-validated TaqMan Gene Expression Assays. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Hs02758991\_g1) and hypoxanthine phosphoribosyltransferase 1 (HPRT1, Hs02800695\_m1) were used as housekeeping genes. The following additional TaqMan Gene Expression assays were used: Hs00219132\_m1 to capture all TREM2 transcripts, a custom assay was designed for *TREM2alt* (assay ID:AI89K96), and Hs00610419\_g1 for *AIF1* (aka *IBA1*). Real-time PCR cycle threshold (CT) raw data was collected and exported using the ABI PRISM SDS software version 2.2. The variable  $\Delta CT$  denotes the difference between the averaged CT values for the *TREM2* transcripts and that for the housekeeping genes. The fold change ( $2^{-\Delta\Delta Ct}$ ) for each sample was calculated relative to the median  $\Delta CT$  in control samples (AD vs control) or subjects homozygous for the major allele (R47H), as relevant.

#### EndoH and PNGaseF treatments

100  $\mu$ g of RIPA-soluble fractions were boiled for 5 min in denaturing buffer. After cooling, the samples were incubated with 2  $\mu$ l of either EndoH (1000U) or PNGaseF (1000U) overnight at 37 °C in their respective assay buffers supplemented with 1% NP-40. Both enzymes and buffers were from New England Biolabs. The whole reaction was loaded on a gel for Western blot analysis.

#### Western blot

Temporal cortexes were extracted with RIPA buffer (Sigma) and soluble fractions were generated by centrifugation for 30 min at 20,000 g. Protein concentration in the RIPA-soluble fractions was determined by BCA assay and 100  $\mu$ g from each sample were run on 4–20% gradient Tris-glycine Novex gels (Invitrogen). Transfer

of the proteins to nitrocellulose membranes was carried out at 70 V for 2 h. The membranes were then blocked for 1 h with 5% milk in TBS 0.05% Tween-20 and incubated overnight with the following TREM2 antibodies: a C-terminal Ab D8I4C (1–500, Cell Signaling) and N-terminal Ab 175262 (1–500, Abcam). The other primaries antibodies used were actin (I-19 1–100, Santa Cruz) and Iba1 (1–2000, Abcam 178847). The blots were washed five times with TBS 0.05% Tween-20 and incubated with an anti-rabbit HRP antibody (1–2000) for 1 h at room temperature. The blots were developed with SuperSignal West Femto reagent (Pierce), imaged with the Fujifilm Luminescent Image Analyzer LAS4000 System, and the bands were quantitated using ImageQuant software.

#### Statistical analysis

Relative expression levels were calculated using the  $2^{-\Delta\Delta Ct}$  method, and tested for differences between groups using the Wilcoxon rank sum test with Prism software. When this test was significant, we used multi-variable linear regression to further assess the association, using  $\Delta Ct$  as the expression variable and adjusting for the following covariates: age at death (years), sex, RNA integrity number (RIN), qPCR plate and measured levels ( $\Delta Ct$ ) of *IBA1*. Statistical analyses were performed using the R Statistical Software (R Foundation for Statistical Computing, version 3.2.3). Prism software was used for statistical analysis of data presented in Figs. 3 and 4.

#### Abbreviations

AD: Alzheimer's disease; A $\beta$ : Amyloid beta; CFT: Carboxy terminal fragment; Ct: Cycle threshold; qPCR: Quantitative real time polymerase chain reaction; sTREM2: Soluble TREM2; TREM2: Triggering receptor expressed in myeloid cells 2; *TREM2alt*: Alternative spliced TREM2

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#### Availability of data and materials

N/A.

#### Authors' contributions

LM carried out RNA extractions and qPCR. MA performed the statistical analysis, and helped with data interpretation and drafting the manuscript. NS provided critical analysis of the data. NET and DWD provided brain samples. DWD was also responsible for postmortem diagnosis. NBR provided clinical diagnosis. SYG and DS conceived the study. DS carried out Western blot analysis, was responsible for data interpretation, and writing the manuscript. All authors read and approved the final manuscript.

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**Competing interests**

The authors declare that they have no competing interests.

**Consent for publication**

All authors critically revised the manuscript and approved the final version before submission.

**Ethical approval and consent to participate**

N/A.

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Continuation of the above referenced study is approved by expedited review procedures (45 CFR 46.110, item 5). This approval is valid for a period of one year. Reviewer noted that study enrollment has been closed. The Reviewer determined the research continues to pose no more than minimal risk to subjects. The Reviewer determined that this research continues to satisfy the requirements of 45 CFR 46.111.

Signed by the Mayo Clinic Institutional Reviewer.

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