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RESEARCH ARTICLE



Transthyretin stabilizer therapy increases naturally-occurring antibodies in ATTR cardiomyopathy

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ABSTRACT

Background: Amyloid transthyretin cardiomyopathy (ATTR-CM) results from extracellular deposition of misfolded transthyretin (TTR), causing progressive heart failure. Naturally-occurring antibodies (nAbs) targeting misfolded proteins exist in neurodegenerative disease, but their presence in ATTR-CM is unknown. The objective of this study is to determine whether nAbs against TTR (nAbs_{TTR}) exist in humans and whether they are influenced by disease or its treatment. **Methods:** Serum from healthy donors, umbilical cord blood (UCB), and patients with ATTR-CM - both untreated and receiving TTR-stabilizing therapy - was analyzed for nAbs_{TTR} using immunoassays, blotting, and binding studies. Functional activity was evaluated in a fibril formation assay.

Results: nAbs_{TTR} binding both native and amyloid TTR (ATTR) with high affinity (KD 30 nM/7 nM) were detected in healthy serum and UCB. nAbs_{TTR} levels were significantly altered in ATTR-CM compared to controls: nAbs_{TTR} (IgG) were higher while nAbs_{TTR} (IgM) were lower. nAbs_{TTR} of both subtypes significantly increased by 22% ($p \leq 0.05$) in patients receiving TTR-stabilizing therapy. *In vitro*, nAbs_{TTR} suppressed TTR fibril aggregation.

Conclusions: Naturally-occurring TTR-targeting antibodies are present from birth, modulated by disease and therapy, and inhibit fibril formation. These findings reveal an unrecognized immune mechanism with potential relevance for ATTR-CM pathogenesis and treatment.

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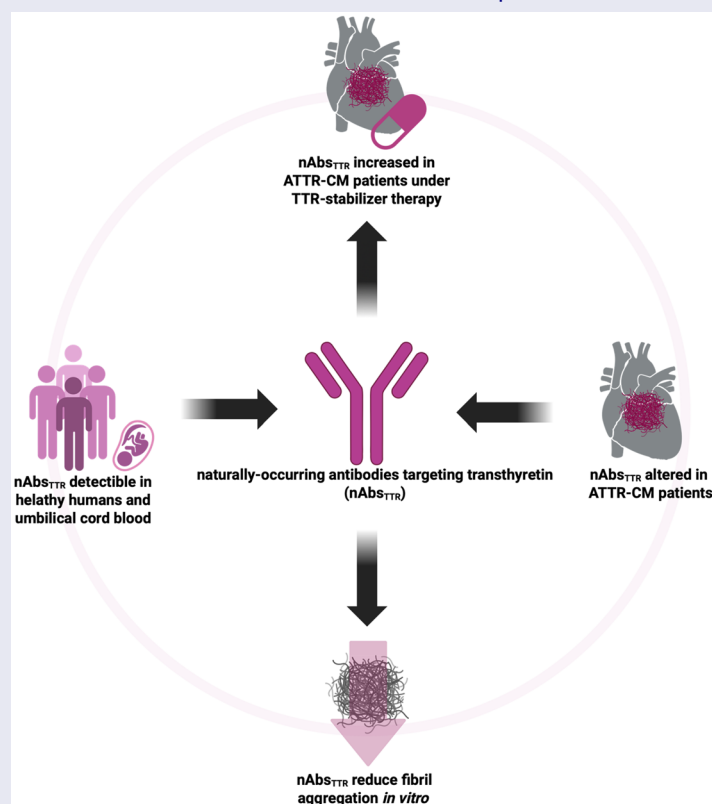
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GRAPHICAL ABSTRACT

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Abbreviations: ATTR: Amyloid Transthyretin; ATTR-CM: ATTR-Cardiomyopathy; IVIG: intravenous immunoglobulins; nAbs: naturally-occurring antibodies; nAbs_{xxx}: nAbs targeting xxx; PENTA: Pentaglobin; TTR: Transthyretin; UCB: umbilical cord blood

Introduction

Cardiac transthyretin amyloidosis (ATTR) is a progressive heart disease caused by myocardial extracellular accumulation of misfolded plasma transthyretin in the heart (TTR). TTR is an extracellular protein of 55 kDa that is primarily produced in the liver and plays a well-established role in transporting thyroxine and retinol [1,2]. Amyloid TTR cardiomyopathy (ATTR-CM) leads to progressive and ultimately fatal heart failure [3]. The majority of cases suffer from the wild-type form of the disease (wtATTR-CM). Until 2025, the only approved treatment to reduce mortality and hospitalizations in patients with ATTR-CM was the TTR stabilizer Tafamidis, which stabilizes the TTR tetramer, thereby inhibiting the formation of amyloidogenic fibril formation from monomers [4–7]. The disease-reversing mechanisms currently under discussion are said to be antibody-induced phagocytosis [8]. High levels of circulating spontaneously occurring polyclonal IgG

antibodies to human TTR amyloid have been described in a small number of patients with self-stabilizing disease activity in the absence of therapy [9–11]. Recombinant human IgG antibodies have been developed for therapeutic use, selectively binding to amyloid conformations and facilitating the depletion of cardiac transthyretin amyloid [12,13].

In contrast to reactively generated or recombinantly produced antibodies, naturally-occurring antibodies (nAbs) are a crucial component of the innate immune system, providing broad-spectrum defense against pathogens and contributing to immune homeostasis [14]. Unlike adaptive antibodies, nAbs are present without prior antigen exposure.

The innate immune repertoire in healthy individuals and ATTR-CM patients has not yet been described. Given conceptual similarities in amyloidogenic diseases, we hypothesized that nAbs against TTR (nAbs_{TTR}) might exist in the plasma of healthy and ATTR-CM diseased humans.

Material and methods

For detailed protocols, see [Supplemental material](#).

TTR ELISA

96-well plates were coated with 50 μ l (2 μ g/mL) of TTR variants (TTR_{wt}: human recombinant transthyretin, Alexotech, Umeå, Sweden; TTR_{V122I}: human recombinant transthyretin variant V122I, Alexotech, Umeå, Sweden; TTR_{nat}: native human prealbumin, Bio-Rad Laboratories, Hercules (CA), USA) and amyloid fibril TTR variants (aTTR_{L55P}: Human Recombinant Transthyretin L55P Variant Filaments, StressMarq Biosciences, Victoria (BC), Canada) or α -synuclein (α Syn, Alexotech, Umeå, Sweden) using sodium bicarbonate coating buffer. Amyloid forms of TTR-variants (aTTR_{wt} and aTTR_{V122I}) have been generated using the fibril formation assay (see corresponding method), initially verified by Thioflavin T (ThT) method as described elsewhere ([Supplemental Figure 1 A](#)) [15]. For ELISA experiments, fibrils were generated in the absence of ThT. Blocking was performed for 2 h at room temperature on a horizontal shaker at 100 rpm using 100 μ l ROTIBlock solution (Carl Roth GmbH, Karlsruhe, Germany). Plates were incubated afterwards with 50 μ l human serum, intravenous immunoglobulin (IVIG) (Octagam 10%, Octapharma, Langenfeld, Germany) or IgM-enriched immunoglobulins (PENTA, Pentaglobin, Biotest, Dreieich, Germany) for 1 h at room temperature and 100 rpm. IVIG is a pharmaceutical preparation of plasma-derived pooled human IgG from healthy blood donors and therefore also contain nAbs [16]. PENTA as an IgM-enriched formulation is available for clinical use [17].

Isolation of nAbs_{TTRiso} from healthy human individuals

NAbs reactive against TTR (nAbs_{TTRiso}) were isolated from IVIG *via* AminoLink Plus affinity chromatography (Thermo Fisher Scientific, Massachusetts, USA). Recombinant proteins were immobilized on columns in coupling buffer with NaCNBH₃, followed by washing and elution with 100 mM glycine (pH 2.8).

Characterization of B-cells

Peripheral blood from healthy donors ($n=10$, female $n=5$, age 19–69) was collected. PBMCs were isolated *via* Ficoll density centrifugation, and CD19⁺ B-cells were enriched using microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and EasySep Magnet (Stemcell technologies, Vancouver, Canada). TTR_{nat}

was conjugated to Cy3/Cy5, and cells were stained with antibodies against CD5, CD20, CD21, CD27, IgG, and IgM (Miltenyi Biotec, Bergisch Gladbach, Germany). Flow cytometry (CytoFLEX) identified mature B-cells (CD19⁺ CD20⁺ [18]). To increase the validity of the yield, TTR double staining was used. B1a-like IgM-expressing and TTR_{nat}-reactive B-cells were defined by the marker constellation CD19⁺ CD20⁺ TTR⁺ IgM⁺ CD5⁺. The representative flow gating strategy used for the detection of TTR-reactive B-cells from human peripheral blood samples is displayed in [Supplemental Figure 2 A](#).

Luminex xMAP® MagPlex® bead-based multiplex immunoassay

Human recombinant TTR_{wt} (Alexotech, Sweden), TTR_{L55P} (StressMarq Biosciences, Canada), and α -synuclein (Alexotech) were each covalently coupled (10 μ g per analyte) to distinct sets of 2×10^6 MagPlex® microspheres (Luminex Corp., USA) using the xMAP® Antibody Coupling Kit according to the manufacturer's instructions. Briefly, beads were activated with EDC (Thermo Fisher Scientific, USA) and Sulfo-NHS and incubated with the respective protein for 2 h at room temperature with rotation in the dark, followed by washing and storage at 4°C until use. Binding of IVIG-derived nAbs_{TTRiso} to coupled microspheres was assessed by incubating 2,500 beads per analyte with 50 μ L nAbs_{TTRiso} (50 μ g/mL in PBS + 1% BSA) for 1 h at room temperature on a plate shaker (800 rpm), with washes between steps. Detection was performed using PE-conjugated anti-human IgG (1:20; BD Biosciences, Cat. No. 560951) incubated 30 min at room temperature in the dark. After a final wash, beads were resuspended in PBS + 1% BSA and analyzed on a LABScan 3D™ Advanced Multiplex Analyzer (Luminex Corp.) with xPONENT software, reporting median fluorescence intensity (MFI) from the PE channel.

Fluorescence dot blotting

Five μ g protein was spotted on nitrocellulose membranes, blocked, incubated with nAbs_{TTRiso}, and detected using IRDye 800CW goat anti-human IgG (LI-COR Biosciences, Lincoln, NE, USA) [19]. Signals were imaged and quantified using Odyssey XF and Empiria Studio software. (LI-COR Biosciences, Lincoln, NE, USA) version: 3.2.0.186.

Microscale thermophoresis (MST)

Cy5-labeled TTR (50 nM) (TTR-Cy5, Cy5 conjugation Kit, Abcam, Cambridge, United Kingdom) was

incubated with serially diluted nAbs_{TTR} (0.01–300 nM) for 14 h at 25°C. MST measurements were performed using Monolith NT.115, and KD values were calculated using MO. Affinity Analysis software (NanoTemper Technologies, Munich, Germany) version 2.1.3.

Surface plasmon resonance spectroscopy (SPR)

Interactions between 6xHis-tagged TTR (Acro Biosystems, Basel, Switzerland) and nAbs_{TTRiso} were assessed on a Biacore 8K (Cytiva, Uppsala, Sweden). 6xHis-tagged TTR was immobilized on a Ni-NTA chip (Xantech, Düsseldorf, Germany). Multi-cycle kinetic experiments were performed with serial dilutions of antibodies (18.875–300 nM), and data were fitted to a 1:1 interaction model.

Fibril formation assay

Fibril formation assay was performed using Thioflavin T (ThT) method as described elsewhere [15,20,21]. Proteins (1 mg/mL) were incubated with 10 µM Thioflavin T, with trypsin added after incubation. Samples were shaken at 500 rpm for 48 h. NAbs_{TTR} were added prior to incubation. Fluorescence was measured at 440/480 nm using CLARIO Star Plus plate reader (BMG Labtech, Ortenberg, Germany).

Transmission electron microscopy

Carbon-coated Formvar films on 200-mesh copper grids (S162, Plano, Wetzlar, Germany) were glow-discharged (PELCO easiGlow™, Ted Pella, Redding, CA, USA) to render the surface hydrophilic. Five µL of sample suspension was applied to each grid for 5 min, washed twice on 30-µL droplets of deionized water, and stained for 1 min on 20-µL droplets of 1% (w/v) aqueous uranyl acetate (Electron Microscopy Sciences, Hatfield, PA, USA). Excess stain was removed with filter paper before air-drying. Images were recorded using a JEOL JEM-1400Plus (120 kV) equipped with a 4096 × 4096 pixel CMOS camera (F416, TVIPS, Gauting, Germany) and EMMENU software (v4.09.83). Image processing and analysis were performed with ImageJ (v1.54p).

Peptide microarray epitope mapping

For this antibody profiling experiment, a library of 34 peptides representing the human transthyretin protein was synthesized and immobilized on microarray slides (JPT Peptide Technologies GmbH, Berlin, Germany; batch #3667). Arrays were processed in a multiwell incubation chamber. Samples of three separately

isolated nAbs_{TTRiso} diluted in blocking buffer (Superblock TBS-T20, Pierce International, Rockford, IL, USA) were applied to the arrays for 1 h at 30°C, followed by incubation with secondary antibody (anti-human IgG, Jackson ImmunoResearch, West Grove, PA, USA; 109-605-098) labeled with AlexaFluor 647 at 1 µg/mL in blocking buffer for 1 h at 30°C. Arrays were washed with 50 mM TBS-T (0.1% Tween-20, pH 7.2) between steps and dried before scanning. Fluorescence signals were acquired using an Axon Genepix 4300 SL50 scanner with constant laser and resolution settings and analyzed using GenePix software. Mean signal intensities (0–65535 AU) were extracted for each spot. MMC2 values were calculated as the mean of triplicate spots unless the coefficient of variation (CV) exceeded 0.5, in which case the mean of the two closest values (MC2) was used. Data processing was performed in Microsoft Excel and R.

Phagocytosis assay

RAW 264.7 cells (ATCC, Manassas, VA, USA) were cultured in DMEM with 10% FBS, 20 mM HEPES, 2 mM Glutamine, and 1% Pen/Strep. (all components from PAN Biotech, Aidenbach, Germany). For the detection of TTR_{nat} and aTTR_{L55P} phagocytosis, both were conjugated with Cy5 using the Cy5 Conjugation Kit-Lightning-Link (Abcam, Cambridge, UK). After incubation of TTR_{nat} or aTTR_{L55P} (7.5, 10 and 15 µg/mL) with nAbs_{TTRiso} (10 and 20 µg/mL) at 37°C for 1 h, the samples were transferred to the cells for 3 h of incubation. Acquisition of stained cells was carried out using the CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA) and analyzed using CytExpert software. After identifying the RAW 264.7 cells and gating singlet cells based on FSC-Width, TTR⁺ cells were detected using the TTR_{nat}-/aTTR_{L55P}-Cy5 signal.

Human samples

PB samples from healthy adults and UCB samples from healthy term neonates were obtained after informed consent according to the Declaration of Helsinki [22] and approval by the ethics committee of the Medical Faculty at the University of Duisburg-Essen (Essen, Germany; 20-9129-BO (UCB) and 10-4380-BO (PB)). For human wtATTR-CM samples from the West German Amyloidosis Center, University Hospital Essen, Germany and from the Interdisciplinary Amyloidosis Center Northern Bavaria, University Hospital Würzburg, Germany, were analyzed. For this multicenter real-world cohort, all consecutive patients presenting with available Biobank samples ≥ 18 years

of age diagnosed with wtATTR-CM were screened for this study. Diagnosis was confirmed *via* ^{99m}Tc -DPD scintigraphy after exclusion of monoclonal gammopathy or *via* endomyocardial biopsy according to international guidelines. The control group of patients with light chain (AL) amyloidosis without cardiac involvement, patients with AA amyloidosis, and CM patients without amyloidosis ($n=16$ (5/8/3) after multivariate adjustment for age and sex (control cohort was intentionally selected to include patients with amyloidosis not involving the heart). This approach aimed to exclude potential pan-amyloid effects and to provide data that go beyond those obtained from a conventional HFpEF cohort, ensuring broader representativeness. Multivariate adjustment was applied to reduce potential confounding, as matching was not feasible due to the retrospective design. Controls were evaluated for ATTR-CM using a guideline-aligned, stepwise approach: Initial clinical assessment and transthoracic echocardiography. Controls without echocardiographic red flags or clinical features suggestive of ATTR-CM did not undergo further testing. If echocardiographic or clinical findings suggested possible cardiac ATTR-amyloidosis or an overlap syndrome, we performed bone-tracer scintigraphy and, where appropriate, excluded AL amyloidosis with serum/urine immunofixation and serum free light chains [23]. Patients with available blood serum samples within 12 months prior and 12 months after the start of TTR stabilizer therapy with Tafamidis were included ($n_{\text{total}} = 181$ patients, 425 consecutive patient visits). The study was approved by the respective local ethics committee (ethics approval 23-11600-BO; 194/22-sc). Human biological samples and related data were provided by the Westdeutsche Biobank Essen (WBE, University Hospital Essen, University of Duisburg-Essen, Essen, Germany; approval 24-WBE-174). Baseline characteristics are summarized in Table 1.

Statistics & visualization

Statistical analyses and visualization were performed using GraphPad Prism version 10.4.1 (GraphPad Software, Boston, MA, USA). Data normality was assessed using the Shapiro-Wilk test. Depending on normality, group comparisons were conducted using unpaired Student's t-tests or Mann-Whitney U tests (two groups), Wilcoxon test in paired samples, and one-way ANOVA with Tukey's post-hoc or Kruskal-Wallis with Dunn's post-hoc (more than two groups). Two-way ANOVA assessed interactions between two independent variables. Pearson's or Spearman's correlation was used for parametric or

non-parametric data, respectively. Corrections for multiple testing were conducted where applicable. Because a subset of patients had more than one measurement, we analyzed antibody levels using linear mixed-effects models with a random intercept for patient to account for within-subject correlation. Statistical significance was set at $p < 0.05$. Data are presented as mean $\pm$ SD or median (IQR), as appropriate. IgG_{nAbs0} levels were log-transformed to correct right-skewness; outliers were removed using the IQR method. Group differences were analyzed using unadjusted and age- and sex-adjusted linear regression models.

Results

Identification of IgG_{nAbs} and IgM_{nAbs} targeting TTR in healthy subjects

The majority of reported nAbs are IgM and IgG subtypes [24]. To identify nAbs targeting TTR, we analyzed plasma from healthy human subjects. UCB is an important resource for nAbs research as IgM populations in cord blood are of neonatal origin and are therefore used to prove their nAbs nature, as vertical transfer of IgG antibodies cannot be excluded [24].

Table 1. Baseline characteristics.

Parameter	Number
N_{total} (consecutive visits)	181 (425)
Male/female (%)	88.9/11.1
Age (years); mean (min/max)	77.9 (57/91)
Mean BMI (kg/m²) (min/max)	26.5 (18.9/43.8)
NYHA I (%)	5.3
NYHA II (%)	50.0
NYHA III (%)	40.3
NYHA IV (%)	4.4
NAC I (%)	37.6
NAC II (%)	45.3
NAC III (%)	17.1
Mean 6-MWD (m) (min/max)	323 (20/594)
Median LVEF (%) (IQR)_{BL/ST}	50.0 (56.6-42.2)/47.3 (55.0-36.7)
Median IVSd (mm) (IQR)_{BL/ST}	18.9 (21.6-15.3)/18.9 (21.6-16.5)
Median LVIDd (mm) (IQR)_{BL/ST}	43.0 (46.6-39.0)/42.9 (49.3-39.0)
Median NT-proBNP (pg/mL) (IQR)_{BL/ST}	3256 (5353-1804)/3618 (8441-2128)
Median cTnl (ng/l) (IQR)_{BL/ST}	40.0 (68.0-25.0)/32.5 (56.3-18.5)
Median nAbsTTRIgM (AU) (IQR)_{BL/ST}	1.61 (2.16-1.13)/1.68 (2.28-1.17)
Median nAbsTTRIgG (AU) (IQR)_{BL/ST}	0.39 (0.55-0.29)/0.38 (0.60-0.31)
Median TTR (mg/dl) (IQR)_{BL/ST}	26.7 (32.0/21.8)/30.2 (34.1-25.4)
Median eGFR (mL/min/1.73 m²) (IQR)_{BL/ST}	59.9 (76.1-41.0)/48.0 (68.1-33.0)
Median S-creatinine (mg/dl) (IQR)_{BL/ST}	1.26 (1.49-10.8)/1.32 (1.71-1.16)

Abbreviations: BMI = body mass index; NYHA = New York Heart Association Dyspnea scale; NAC = National Amyloidosis Center Stage [48]; 6-MWD = six-minute walking distance; LVEF = left ventricular ejection fraction; BL = baseline; ST = under stabilizer therapy; IVSd = intraventricular septum diastolic; LVIDd = left ventricular inner diameter diastolic; NT-proBNP = N-terminal pro brain natriuretic peptide; cTnl = cardiac troponin I; nAb = naturally-occurring antibodies; TTR = transthyretin, eGFR = estimated glomerular filtration rate, S = serum.

PENTA is an IgM-enriched IVIG formulation of plasma-derived immunoglobulins from healthy blood donors and therefore also contains nAbs [16,17]. Significant levels of IgG_{nAbs} were detected against TTR_{wt}, TTR_{V122I} and TTR_{nat} in UCB and IVIG, with the elevated IVIG-levels indicating a higher antibody concentration due to pooled plasma concentration (Figure 1 A-C). Level of IgM_{nAbs} presented similar properties in comparison to PENTA levels (Figure 1 G-I).

IgG_{nAbsTTR} and IgM_{nAbsTTR} recognize ATTR

To determine whether the fibrillar amyloidogenic form of TTR (ATTR) is also recognized by nAbs_{TTR} in serum and IVIG formulations, ATTR fibril aggregates were tested. Significant binding of IgG and IgM was detected in aTTR_{wt}, aTTR_{V122I} and aTTR_{L55P}. Thus, even altered epitopes were still recognized by nAbs_{TTR} (Figure 1 D-F, J-L, Supplemental Figure 1 B).

Affinity of IgG_{nAbs} and IgM_{nAbs} targeting (A)TTR

A step-up approach was used to test the affinity of the identified nAbs_{TTR} for their target protein. First, the basal binding capacity was tested at the protein level using ELISA and dot blotting. The nAbs_{TTRiso} isolated from IVIG exhibited significantly higher binding for TTR_{wt} and aTTR_{L55P} than for α -synuclein or Amyloid- β , which served as amyloid control proteins. This confirms successful binding. (Figure 2 A). Compared to IVIG, nAbs_{TTRiso} isolation resulted in an increase in concentration of nAbs_{TTRiso}, demonstrating successful isolation (Supplemental Figure 1 B). A low threshold signal was detected in α -synuclein binding, likely attributable to nonspecific binding inherent in nAbs. After demonstration of successful binding, we assessed the affinity of nAbs_{TTRiso}, by performing SPR. While IVIG with nAbs_{TTR} showed a K_D of 21.4 μ mol/L (Figure 2 B), nAbs_{TTRiso} concentrated from IVIG showed a strong affinity with a K_D of 558 nmol/L (Figure 2 C). Binding was detectable in MST experiments on PENTA as well (see supplement, Supplemental Figure 1 C, D). Control nAbs_{aSyn} showed no binding to TTR_{nat} in MST and low binding in SPR (see supplement, Supplemental Figure 1 E-G). To further demonstrate the affinity of nAbs_{TTRiso}, we applied a microscale thermophoresis assay (MST). Here, we found high affinity binding of nAbs_{TTRiso} to TTR (K_D = 30 nM) (Figure 2 D, E) as well as to aTTR_{L55P} (Figure 2 F, G). Affinity measurements varied depending on the method used, with MST showing a K_D of 30 nM and SPR showing a K_D of 558 nM, consistent with high-affinity binding in both systems.

Identification of TTR_{nat}-producing B-cells

For identification of TTR_{nat}-producing B-cells, we analyzed donor blood from healthy donors using Fluorescence-Activated Cell Sorting (FACS) analysis. Populations were defined based on the expression of CD19, CD20, CD5, CD27, IgG and IgM. The expression of CD19⁺ and CD20⁺ on mature B-cells allowed the identification of double-positive TTR_{nat}-Cy3⁺ and TTR_{nat}-Cy5⁺ as TTR-reactive B-cells (TTR⁺). Among these, we further characterized CD27⁺ memory- and CD5⁺ B1a-like subsets. Further subsets were identified by the expression of IgG (TTR⁺ IgG⁺) or IgM (TTR⁺ IgM⁺), including IgG⁺ CD27⁺, IgM⁺ CD27⁺ and IgM⁺ CD5⁺ subset populations, potentially indicating innate-like or unswitched memory B-cell phenotypes. These data suggest a diverse TTR-reactive B-cell repertoire, hinting toward the B1a-like nature of TTR-reactive B-cells (Figure 2 H-K).

A multicenter study was conducted in a real-world cohort aiming to further validate the findings and to evaluate the relevance and potential implications of the identified nAbs in ATTR-CM:

nAbs_{TTR} homeostasis is altered in ATTR-CM

To assess the prevalence of nAbs_{TTR} levels in patients with diagnosed ATTR-CM, 181 patients (88.9% male, mean age 77.9 years) in 425 cumulative consecutive patient visits were analyzed (baseline characteristics see Table 1). We compared their respective titers to a pooled control (baseline characteristics see Table 2). It has to be noted that increased septal wall thickness in control subgroup AA amyloidosis reflects secondary hypertrophy due to systemic arterial hypertension on the background of the underlying disease; in AL amyloidosis, two patients had hypertensive heart disease with negative NT-proBNP; additionally, one control was an athlete with a history of testosterone substitution.

In direct comparison, we found a significantly lower level of nAbs_{TTR} of the IgM isotype in patients diagnosed with ATTR-CM compared to those without ATTR-CM (Figure 3 A). In ATTR-CM patients without TTR stabilizing treatment, a higher level of nAbs_{TTR} type IgG (IgG_{nAbs}) was detected, when compared to controls (Figure 3 B). Notably, more than half (59.5%) of the titers observed in ATTR-CM patients exceeded one SD of the control group, and 40.5% surpassed two SDs of the control group. There was no association of nAbs_{TTR} level and age detected. Participants did not differ by center on the antibody results.

Transthyretin stabilization is an established therapeutic approach for ATTR-CM [25]. In our cohort,

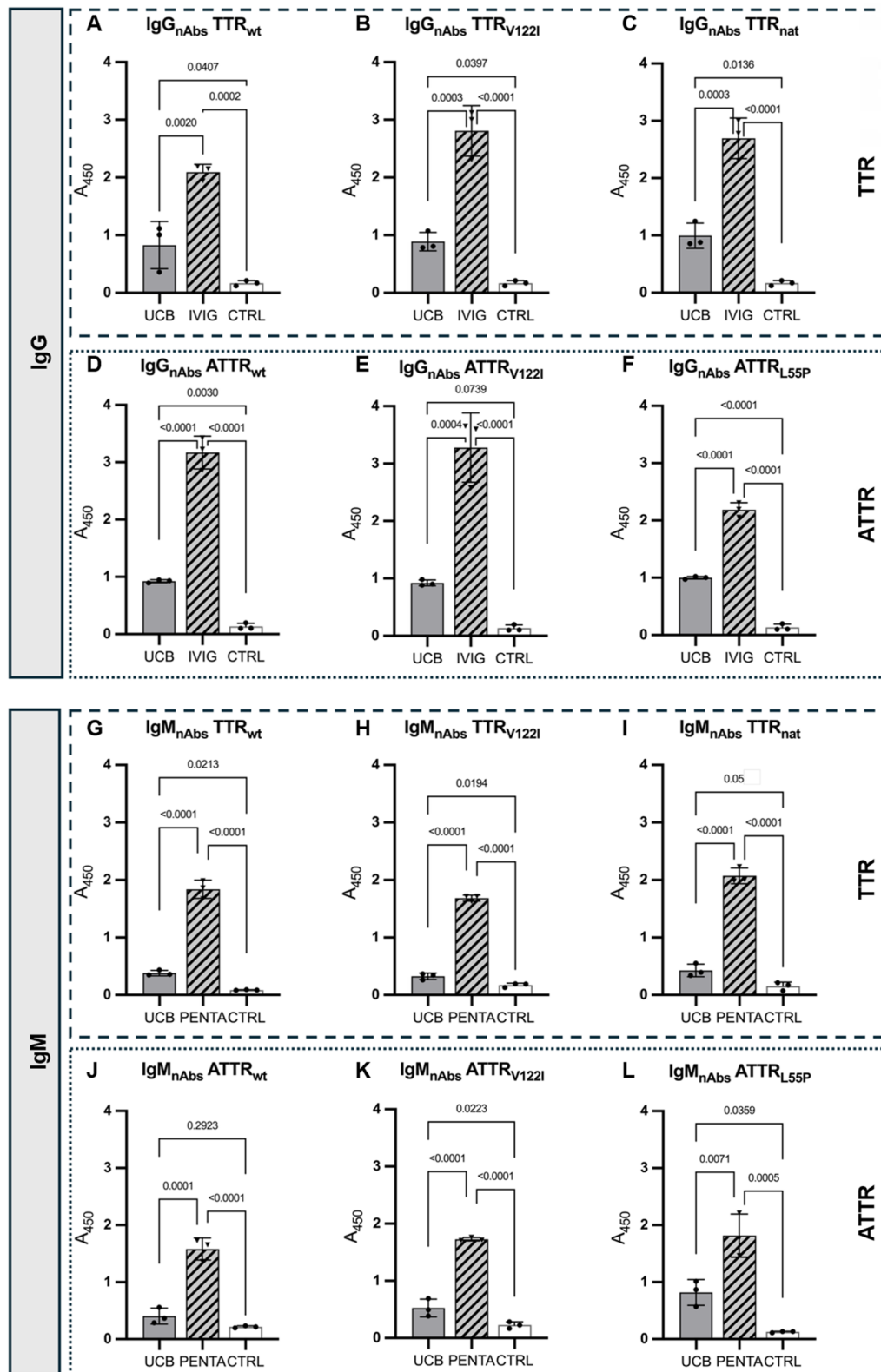


Figure 1. Identification of nAbs_{TTR}. Significant levels of nAbs_{TTR} of IgG-type and IgM-type were detected in human UCB pooled IVIGs or IgM-enriched immunoglobulin solutions (PENTA) of healthy donors. **A–C** Significant titers of IgG_{nAbs} against TTR_{wt}, TTR_{V122I} and TTR_{nat} were detected in UCB and IVIG. **D–F** IgG_{nAbs} against TTR recognize amyloid variants (aTTR_{wt}, aTTR_{V122I}, aTTR_{L55P}) of target protein. **G–I** Significant titers of IgM_{nAbs} against TTR_{wt}, TTR_{V122I} and TTR_{nat} were detected in UCB and PENTA. $n=3$ per condition. Normality test passed, ordinary one-way ANOVA.

Abbreviations: nAbs=naturally-occurring antibodies, TTR=transthyretin, UCB=umbilical cord blood, IVIG=intravenous immunoglobulin, TTR_{wt}=human recombinant transthyretin, TTR_{V122I}=human recombinant transthyretin variant 122I, TTR_{nat}=native human prealbumin, CTRL=technical control, aTTR_{wt}=human recombinant transthyretin filaments, aTTR_{V122I}=human recombinant transthyretin variant 122I filaments, aTTR_{L55P}=human recombinant transthyretin L55P variant filaments, PENTA=Pentaglobin=IgM-enriched IVIG. IgM_{nAbs} and IgG_{nAbs} are displayed in artificial units.

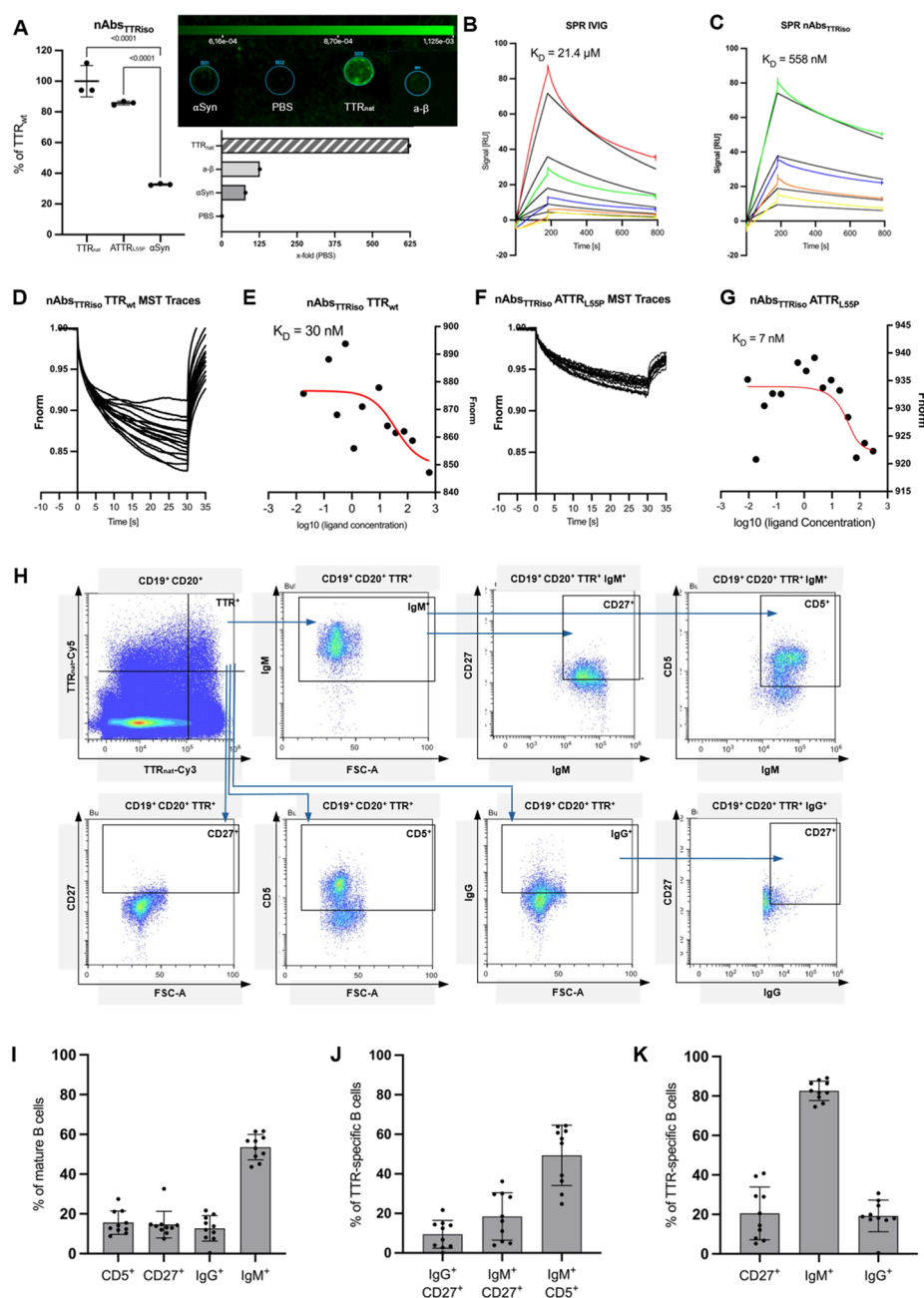


Figure 2. nAbs_{TTRiso} recognize ATTR and its precursor protein with high affinity. **A** nAbs_{TTRiso} present significantly higher binding to TTR_{nat} and aTTR_{L55P} than to αSyn and αβ. *n*=3, ordinary one-way ANOVA. Representative qualitative dot blot imaging. **B-C** MST: nAbs_{TTRiso} bind TTR_{nat} with high affinity ($K_D = 30$ nM). **D-E** MST: nAbs_{TTRiso} bind aTTR_{L55P} with high affinity ($K_D = 7$ nM). **F** IVIG bind TTR_{nat} ($K_D = 21.4$ μM). **G** concentrated purified nAbs_{TTRiso} show strong high-affinity binding ($K_D = 30$ nM). **H-L** SPR: sensorgrams fitted to a 1:1 interaction model (where applicable). Antibodies were diluted from 300 nmol/L to 18.875 nmol/L with a 1:3 dilution series. Injected concentrations are 300 nmol/L (red), 150 nmol/L (green), 75.5 nmol/L (blue), 37.5 nmol/L (orange), 18.875 nmol/L (yellow). All fitted curves are shown in black. **H** Representative flow gating strategy used for the detection of TTR-reactive B-cells from human peripheral blood samples. Individual populations were defined based on the expression of CD19, CD20, CD5, CD27, IgG and IgM. The expression of CD19+ and CD20+ on mature B cells allowed the identification of double positive TTR_{nat}-Cy3 and TTR_{nat}-Cy5 as TTR-reactive B cells (TTR+), CD27+ memory- and CD5+ B1a-like cells. Further subsets were identified by the expression of IgG (TTR+ IgG+) or IgM (TTR+ IgM+), including IgG+CD27+, IgM+CD27+ and IgM+CD5+ subset populations. **I-L** Histograms show the percentages of individual populations: CD5+, CD27+, IgG+, IgM+, CD19+ CD20+ TTR+; TTR+ CD27+, TTR+ IgG+, TTR+ IgM+, TTR+ IgG+ CD27+, IgM+ CD27+, IgM+ CD5+ and TTR-reactive population from CD19+ CD20+ mature B cells (*n* = 10).

Abbreviations: nAbs=naturally-occurring antibodies, TTR=transthyretin, TTR_{nat}=native human prealbumin, aTTR_{L55P}=human recombinant transthyretin L55P variant filaments, αSyn=α-synuclein, αβ=Amyloid-β, MST=microscale thermophoresis, K_D =dissociation constant, SPR=surface plasmon resonance spectroscopy, IVIG=intravenous immunoglobulins

Table 2. Baseline characteristics control group.

Parameter	Number
N_{total} (AL w/o heart, AA w/o heart, HFpEF)	16
AL w/o heart	5
AA w/o heart	8
HFpEF	3
Male/female (%)	56/44
AL w/o heart	60/40
AA w/o heart	55/45
HFpEF	25/75
Age (years); mean (min/max)	69.2 (48/76)
AL w/o heart	62.7 (54/73)
AA w/o heart	63.3 (48/76)
HFpEF	70.3 (60/76)
Mean LVEF (%) (min/max)	58 (49/70)
AL w/o heart	62.2 (54/70)
AA w/o heart	57.4 (49/65)
HFpEF	58 (55/60)
Mean IVSd (mm) (min/max)	16.7 (8/19)
AL w/o heart	12.7 (9/19)
AA w/o heart	11.9 (11/15)
HFpEF	8.7 (8/10)
Mean NT-proBNP pg/mL (min/max)	2179 (18/14478)
AL w/o heart	165 (18/678)
AA w/o heart	4745 (367/14478)
HFpEF	219 (31/460)
Mean cTnI (ng/l) (min/max)	36.3 (3.2/72.5)
AL w/o heart	12.1 (3.2/26.4)
AA w/o heart	35.3 (15.9/72.5)
HFpEF	18.5 (14.4/22.2)
Mean nAbsTTRIgM (AU) (min/max)	2.11 (0.26/2.99)
AL w/o heart	1.44 (0.26/2.96)
AA w/o heart	2.27 (1.2/2.99)
HFpEF	1.94 (1.24/2.48)
Mean nAbsTTRIgG (AU) (min/max)	0.26 (0.17/0.38)
AL w/o heart	0.32 (0.25/0.38)
AA w/o heart	0.25 (0.17/0.31)
HFpEF	0.23 (0.19/0.27)
Mean TTR (mg/dl) (min/max)	27.4 (20.2/35.6)
AL w/o heart	27.9 (20.2/35.6)
AA w/o heart	n/a
HFpEF	30.0 (22.2/35.6)
Mean eGFR (mL/min/1.73 m²) (min/max)	63.3 (17.4/97.8)
AL w/o heart	59.4 (31.9/97.8)
AA w/o heart	31.0 (17.4/50.1)
HFpEF	56.5 (47.3/62.7)
Mean S-creatinine (mg/dl) (min/max)	1.50 (0.66/2.14)
AL w/o heart	1.32 (0.66/2.14)
AA w/o heart	2.02 (1.63/2.58)
HFpEF	1.20 (1.12/1.32)

Abbreviations: LVEF=left ventricular ejection fraction; IVSd=intraventricular septum diastolic; NT-proBNP=N-terminal pro brain natriuretic peptide; cTnI=cardiac troponin I; nAb=naturally-occurring antibodies; TTR=transthyretin, eGFR=estimated glomerular filtration rate, S=serum.

patients receiving Tafamidis showed significantly higher circulating TTR levels within the first year of therapy, compared to both pretreatment levels and baseline at therapy initiation (Figure 3 C). To assess the impact of Tafamidis therapy on nAbs levels, we compared serum samples from two groups: one receiving Tafamidis and one without disease-modifying therapy. We analyzed nAbs_{TTR} titers at initial diagnosis and within 12 months of either natural disease progression or treatment. Among untreated patients, 31.4% showed a relative increase in IgM_{nAbs} levels. In contrast, 66.4% of ATTR-CM patients receiving Tafamidis therapy exhibited an increase in IgM_{nAbs} titers during the observation period, indicating a

significantly higher proportion compared to untreated patients (Figure 3 D). IgG_{nAbs} showed a similar pattern of behavior (Figure 3 E). Notably, the relative increase of nAbs_{TTR} did not strongly correlate with TTR levels, suggesting a weak association and increase independent of TTR levels (Figure 3 F, $r = -0.18$, Supplemental Figure 1 H). The absolute values are subject to inter-individual variation. In the context of Tafamidis therapy, a substantial increase in IgM_{nAbs} titers is observed in absolute terms (Figure 3 G). Despite inter-individual variability, the absolute IgM_{nAbs} titers increased significantly in the treated group (Figure 3 G), while IgG_{nAbs} titers showed a non-significant upward trend (Figure 3 H). Figure 3 I depicts the

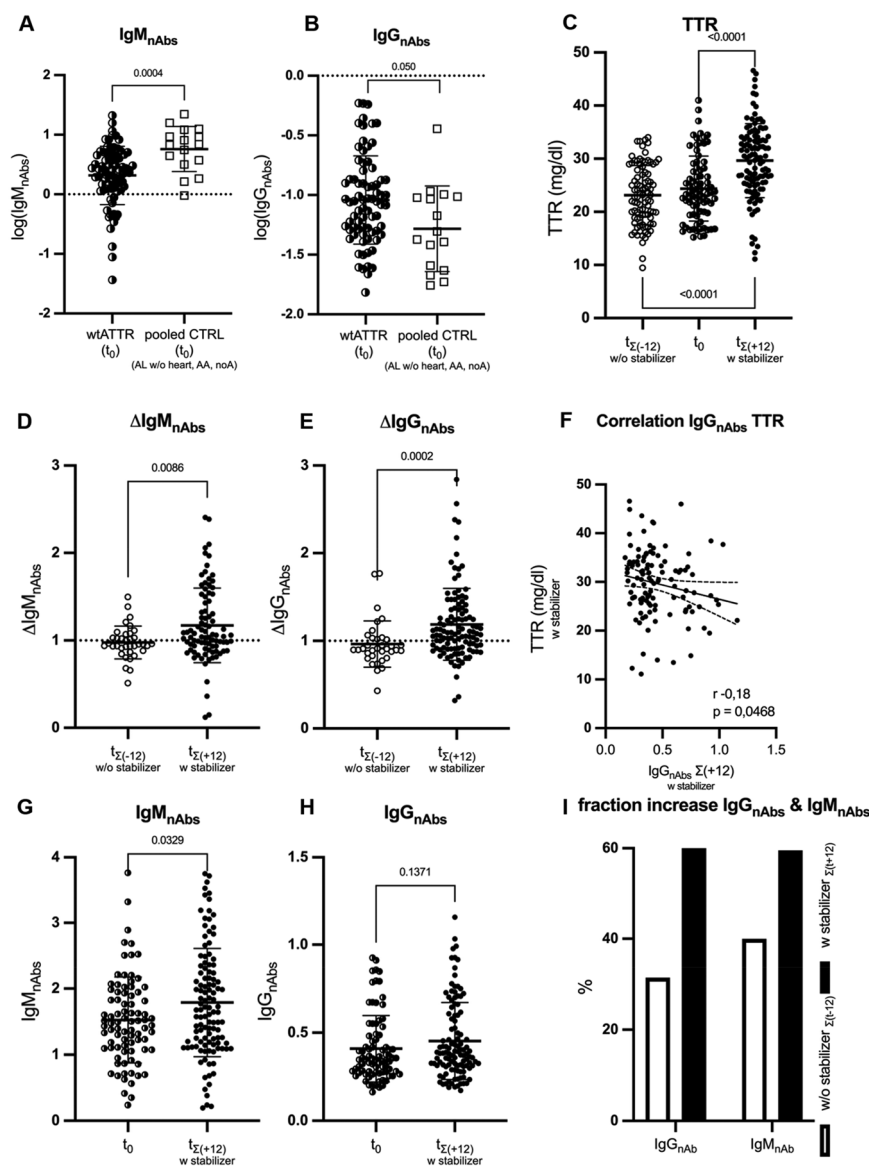


Figure 3. $nAbs_{TTR}$ in wtATTR-CM. $nAbs_{TTR}$ levels have been analyzed in patients with wtATTR-CM. **A** $\lg M_{nAbs}$ are lower in untreated wtATTR-CM patients compared to pooled control data of patients with AL-amyloidosis without heart affection, patients with AA amyloidosis and HFpEF patients. Data adjusted for age and sex. n (wtATTR) = 81, n (pooled CTRL) = 16 (5/8/3), multiple linear regression analysis, p -value = 0.0004. **B** $\lg G_{nAbs}$ are lower in untreated wtATTR-CM patients compared to pooled control data of patients with AL-amyloidosis without heart affection, patients with AA amyloidosis and HFpEF patients. Data adjusted for age and sex. n (wtATTR) = 74, n (pooled CTRL) = 16 (5/8/3), multiple linear regression analysis, p -value = 0.050. **C** TTR stabilizer therapy with Tafamidis increases serum TTR level. TTR levels of pooled patient visits without stabilizer therapy within one year before initiation of therapy ($t_{(-12)}$), at the time point of therapy initiation (t_0) and within one year on stabilizer therapy ($t_{(+12)}$) are displayed. n ($t_{(-12)}$) = 90, n (t_0) = 80, n ($t_{(+12)}$) = 110. Normality test not passed, Kruskal-Wallis test, p -values < 0.0001. **D+E** TTR-stabilizing therapy with Tafamidis results in a significant relative increase in $\lg M_{nAbs}$ and $\lg G_{nAbs}$ based on the first available pretreatment measurement within one year. n ($t_{(-12)}$) = 36 ($\lg M_{nAbs}$)/35 ($\lg G_{nAbs}$), n ($t_{(+12)}$) = 35 ($\lg M_{nAbs}$)/112 ($\lg G_{nAbs}$), normality test not passed, Mann-Whitney test, p -value = 0.0086/ p =0.002. **F** Increase of $\lg G_{nAbs}$ is independent of TTR level. n =111. Normality test not passed, Spearman correlation, r =-0.1892, p -value 0.0468. **G+H** Absolute increase of $\lg M_{nAbs}$ and $\lg G_{nAbs}$ in wtATTR-CM patients with stabilizer therapy compared to initial measurement at treatment initiation. $\lg M_{nAbs}$ are absolutely increased in wtATTR-CM patients, the increase of $\lg G_{nAbs}$ in patients with stabilizer therapy does not reach significant level. n (t_0) = 84 ($\lg M_{nAbs}$)/83 ($\lg G_{nAbs}$), n ($t_{(+12)}$) = 113 ($\lg M_{nAbs}$)/113 ($\lg G_{nAbs}$), normality test not passed, Wilcoxon test, p -value = 0.0329/0.1371 (ns). **I** Fraction increase of $\lg G_{nAbs}$ and $\lg M_{nAbs}$. During the natural course of the disease, a relative increase in $\lg G_{nAbs}$ and $\lg M_{nAbs}$ occurs in less than 40% (314% ($\lg G_{nAbs}$)/40% ($\lg M_{nAbs}$)) of patients. Under TTR-stabilizing therapy, an increase is found in well over half of the patients studied (64% ($\lg G_{nAbs}$)/59% ($\lg M_{nAbs}$)).

Abbreviations: $nAbs$ =naturally-occurring antibodies, TTR=transthyretin, wtATTR-CM=wildtype transthyretin amyloidosis cardiomyopathy, AL=amyloid light chain, AA=serum amyloid A, noA=HFpEF=heart failure with preserved ejection fraction, t_0 = start of therapy, CTRL=control, w/o=without, w=with, ns=not significant, $\lg M_{nAbs}$ and $\lg G_{nAbs}$ are displayed in artificial units.

relative fractional increases in $nAbs_{TTR}$ levels with an overall increase of more than 60% in both groups.

Binding of $nAbs_{TTRiso}$ to C-terminal residues inhibits TTR-fibril formation in vitro but does not affect macrophage phagocytosis

To gain insight into a possible underlying mechanism suggested by these data, $nAbs$ were analyzed in the context of fibrillogenesis in a fibril formation assay. Adding $nAbs_{TTRiso}$ to the fibrillation reaction in the ThT-assay inhibited the fibril formation of both, $aTTR_{V122I}$ and $aTTR_{wt}$, significantly, as indicated by a significantly lower ThT fluorescence intensity (Figure 4 A-C, $p < 0.05$; Supplemental Figure 1 I). Epitope mapping revealed that $nAbs_{TTRiso}$ bind to the Cterminal residues 85–111 of wild type transthyretin, corresponding to the sequence VEGIYKVEIDTKSY WKALGISPFHEHA (Figure 4 D). To investigate whether the presence of $nAbs_{TTR}$ has an effect on ATTR clearance by macrophages independent from the described non-antibody mediated endocytosis [10], a phagocytosis assay was performed using RAW 264.7 cells. RAW 264.7 cells were used as a suitable system for mechanistic investigations of antibody-modulated phagocytosis [26]. The analysis showed no impairment of phagocytosis by the presence of $nAbs_{TTRiso}$ (Figure 4 E), suggesting that these antibodies inhibit fibrillogenesis without affecting macrophage uptake [8,13,27,28].

Discussion

This study provides the first evidence of the existence of autoreactive $nAbs$ against both TTR and ATTR. $nAbs$ of both investigated forms were present in plasma samples of healthy individuals and in IG-enriched formulations and showed a different pattern in patients with ATTR-CM. TTR stabilizer therapy was associated with increasing $nAbs_{TTR}$ titers in patients with ATTR-CM. These findings suggest that $nAbs$ may play a regulatory or diagnostic role in the pathophysiology of ATTR-CM.

The role of $nAbs$ in neurodegenerative protein deposition diseases, like Alzheimer's (Amyloid- β) and Parkinson's disease (α -synuclein), is well established [14,29–31]. Furthermore, particularly natural IgM, are increasingly recognized as modulators of atherosclerosis and cardiovascular disease but rarely shows linear correlations with the circulating concentrations of its target proteins [32]. One of such cases is atherosclerosis, where natural IgM antibodies recognizing oxidation-specific epitopes are generally associated

with reduced plaque burden and lower cardiovascular risk, whereas low levels of these antibodies predict incident cardiovascular events [33]. Importantly, the existence of $nAbs$ in ATTR-CM was yet unknown. Furthermore, such $nAbs$ targeting Amyloid- β or α -synuclein were also found in healthy asymptomatic individuals. Based on the conceptual similarity, we hypothesized that $nAbs$ against TTR might also exist in healthy individuals. We identified IgG type TTR binding antibodies in IVIG formulations. Due to the placental permeability of IgG, it is theoretically possible that IgG antibodies could be passed on by a mother with previous antigen contact. The detection of $nAbs_{IgM}$ in UCB is a strong indicator that these antibodies are naturally-occurring. By definition, $nAbs$ are present without prior antigenic stimulation and can be detected at birth. Although their abundance in umbilical cord blood is lower compared to adult samples, their presence supports the conclusion that the antibodies identified in our study are indeed $nAbs$. Therefore, the inclusion and measurement of umbilical cord blood samples were essential to substantiate the likelihood of these anti-TTR antibodies being of naturally-occurring origin.

Recombinant proteins and native (non-recombinant) TTR proteins were used to confirm that the findings were not limited to a single tested form. Both $nAbs_{IgG}$ and $nAbs_{IgM}$ showed broad reactivity to TTR in various forms, not only against native TTR (TTR_{nat}), but also binding to the corresponding ATTR forms. Given the conservative nature of substitutions, the identified $nAbs$ are likely to recognize multiple TTR variants and are not expected to be strongly affected by single amino acid substitutions like valine to isoleucine substitution in $aTTR_{V122I}$ [34]. Binding was verified by additional independent methods. In addition to fluorescence dot blot binding, affinity studies in both MST and SPR confirmed strong binding of $nAbs_{TTRiso}$. With nanomolar-range dissociation constant, there was no significant difference in affinity between the ATTR form and the native TTR, indicating that fibril formation does not lead to antigen escape. A bead-based multiplex immunoassay further underlined the findings.

To investigate the underlying B-cell repertoire, we performed flow cytometry. In our cohort, B-cell subset frequencies were in normal range, suggesting no overt evidence of chronic stimulation or immunodeficiency in this cohort. It is therefore unlikely that these factors significantly influenced the immune profiles observed. We identified 20% TTR^+ $CD27^+$ and 20% TTR^+ IgG^+ indicating a functional and affinity mature response to TTR. The high proportion of

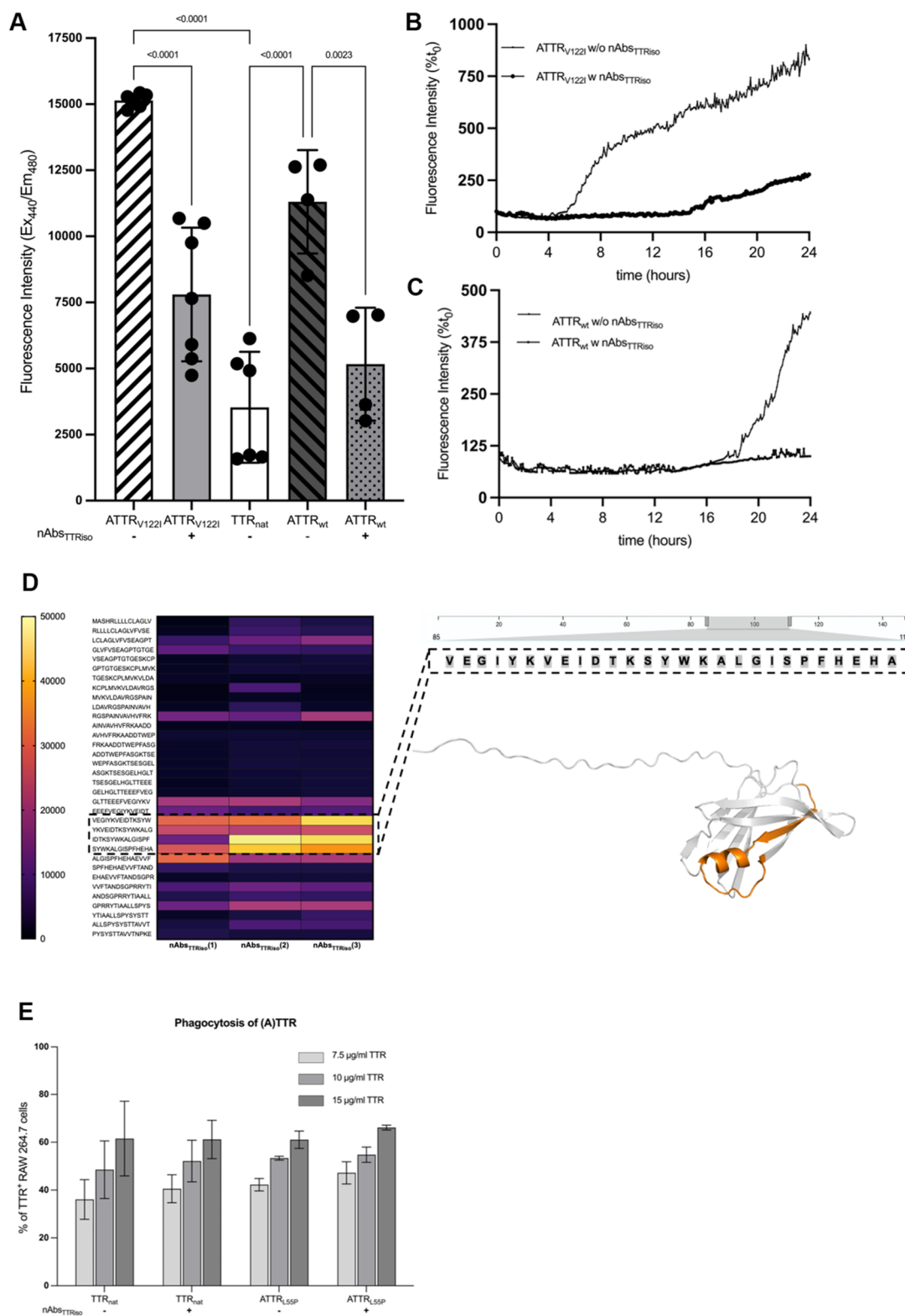


Figure 4. Functional role of nAbs_{TTR}. Presence of nAbs_{TTRiso} inhibits TTR-fibrillation in-vitro. **A** Significant inhibition of fibrillogenesis of aTTR_{v122i} and aTTR_{wt} as demonstrated by lower fluorescence intensity in ThT fibril formation assay. TTR_{nat} served as non-amyloid control in this case. Kinetic data of nAbs_{TTRiso} inhibition of TTR-fibrillation in-vitro early during fibril formation. **B** Inhibition of fibrillogenesis of aTTR_{v122i}. **C** Inhibition of fibrillogenesis of aTTR_{wt}. nAbs_{TTRiso} bind to C-terminal sequence of TTR_{wt}. **D** Epitope mapping showed binding to sequence VEGIYKVEIDTKSYWKALGISPFHEHA of human TTR_{wt}. Presence of nAbs_{TTRiso} did not affect macrophage clearance. **E** Histogram shows the percentages of singlet TTR⁺ macrophages after incubation with TTR_{nat} or aTTR_{L55P}-Cy5 (7.5, 10 and 15 µg/mL target protein) and 10 µg/mL nAbs_{TTR}. Differences not significant. Abbreviations: nAbs=naturally-occurring antibodies, TTR=transthyretin, aTTR_{v122i}=human recombinant transthyretin variant 122I fibril, aTTR_{wt}=human recombinant transthyretin filaments, ThT=Thioflavin T, TTR_{nat}=native human prealbumin, TTR_{L55P}=human recombinant transthyretin L55P variant

IgM⁺ suggests that the majority of TTR-reactive cells in the healthy cohort studied have not undergone class switching. It is also possible that, depending on CD27⁺ status, many TTR-reactive B-cells have not undergone class switching and remain in an early stage of differentiation and have not fully undergone affinity maturation. However, some might represent IgM⁺ memory B-cells, which can be affinity-matured despite lacking class switching. The distribution of TTR⁺ IgM⁺ CD27⁺ B-cells indicates that part of the immune response is long-lived but remains without class switching. The proportion of TTR⁺ IgG⁺ CD27⁺ is slightly lower, indicating that the immune response of healthy individuals is more in the intermediate or early stage and that slightly more TTR-reactive B-cells are not in a classical affinity-matured germinal center response. Next, B1a- and B1b-cells, which in mice are commonly distinguished by the expression of CD5 [35–37], were investigated. They are described to have innate-like functionality and secrete nAbs independent of stimulation [37]. While the existence of true B1a-equivalents in humans remains debated, CD5⁺ IgM⁺ B-cells have been proposed as B1a-like based on surface marker similarities. Thus, a large proportion of the TTR-reactive B-cells with IgM⁺ and CD5⁺ phenotype may be considered B1a-like type, which is consistent with the phenotype of B1-cells described in murine models and in some human studies. These cells are likely contributors to the production of naturally-occurring nAbs. The predominance of IgM⁺-reactive B-cells in healthy donors supports the classification of these antibodies as nAbs as had been shown before [38,39].

Following the identification of nAbs in the healthy cohort, ATTR-CM patients were analyzed to determine whether the antibody repertoires of ATTR-CM patients contain nAbs that can potentially be used as disease markers.

The significant changes (decreased IgM-type and increased IgG-type nAbs) in wtATTR-CM patients compared to the pooled non-ATTR-CM cohort suggest an immunologic component to the disease. It has to remain unclear whether this is a causative or a reactive change. Due to the lack of B-cell analysis in the patients studied, a specific class switch cannot be confirmed but may have occurred. Further maturation or class switching could lead to a response. Due to the retrospective study design, it remains unclear at this time whether these changes precede the disease and thus represent a pathophysiologic cause or a consequence of the disease. However, lack of correlation suggests a causative change. Consistent with data from the ATTR-ACT study [4,40,41], stabilizer therapy

resulted in an absolute and relative increase in total TTR. With stabilizer therapy, we found a significant relative increase in both IgM-type and IgG-type nAbs independent of the increase in total TTR. The increase in nAbs despite constant TTR levels suggests a concentration-independent effect, arguing against a direct causal relationship between increased circulating TTR and the development or amplification of these antibodies. Other studies of in contrast spontaneously occurring antibodies in ATTR-CM patients have not analyzed changes over time but have compared absolute levels of IgG antibodies [10]. The proportion of ATTR-CM patients with increased nAbs during therapy is notable, not only due to the significant inter-group difference, but also due to the individually high titer elevations under therapy.

The crucial difference to the concept of spontaneously occurring antibodies is that the identification of nAbs in healthy individuals suggests a physiological role, possibly in the balancing of proteins and inhibition of fibrillogenesis [9,10]. In disease, they appear to be a reaction caused by the secretion of preexisting antibodies. Thus, it is not only a high level of TTR-targeting antibodies with possible catalytic properties that is important, but especially the relative progression after the start of therapy. As we showed that presence of nAbs did not show any effect on phagocytosis under the conditions tested, the possible mechanism of action is more likely to be found in the reduced aggregation of ATTR. We found that the presence of nAbs clearly inhibited the formation of TTR fibrils. Epitope mapping revealed that nAbs_{TTR} bind to the C terminal residues 85–111 of wild type transthyretin (TTR_{wt}, 147 amino acids), corresponding to the sequence VEGIYKVEIDTKSYWKALGISPFHEHA. This segment spans β sheet-forming regions with steric zipper propensities and contains sequence elements critical for TTR stability as well as for amyloidogenic TTR (ATTR) formation [1]. Although part of this region is partially buried within the native tetramer [2], nAbs_{TTR} recognized all investigated forms of TTR. This broad detection capability may be attributable to the relatively long contiguous sequence recognized. Notably, the epitope targeted by PRX004/Coramitug—a monoclonal antibody drug currently in clinical trials—is encompassed within the sequence recognized by nAbs_{TTR} [3–5]. In contrast to certain engineered antibodies that display conformation specific detection (cryptotope recognition), nAbs_{TTR} appear to detect TTR independently of its conformation, demonstrating a pan-TTR binding property. This observation suggests a possible pathophysiologic role

for nAbs_{TTR} through interaction with transthyretin—a highly conserved protein with numerous physiological functions [6]. Even under physiological conditions, TTR undergoes measurable, albeit extremely slow, dissociation into monomers [1,42–45]. This process alone does not typically result in amyloid disease. The presence of nAbs may represent a molecular protective mechanism, contributing to proteostatic regulation not only by targeting dissociated or misfolded TTR species, but also by binding to the native tetrameric form - potentially stabilizing it and preventing pathological unfolding. Further investigation is warranted to elucidate the precise role of nAbs in maintaining TTR homeostasis and preventing aggregation-related pathology.

This study has limitations. Due to its retrospective nature and limited correlations and associations, no predictive power for nAbs and their dynamics can be established. The approach may introduce certain biases, including availability bias due to reliance on stored samples, potential selection bias related to biobank participation, and survivorship or follow-up bias, as patients with missing samples or early drop-out could not be included. A large number of recombinant and native precursor proteins were examined, but no original isolated patient fibrils could be used. However, this is not a serious drawback in view of possible individual differences in fibril characteristics and the use of both recombinant and native precursor proteins still enabled evaluation of antibody reactivity across a range of conformational states [46]. Owing to sample limitations inherent to the biobank material, retrospective B-cell subpopulation analyses were not feasible. This limitation prevents confirmation of a potential class switch and leaves the question of causality unresolved. This needs to be investigated in a future study including investigation of broader therapeutic approaches. Due to the limited data set, it was also not possible to make a valid statement about the individual course of the disease. A major strength is the multicenter real-world cohort of patients.

In conclusion, this is the first study to describe the dynamics and class distribution of nAbs in ATTR-CM patients and healthy controls, particularly in the context of stabilizer therapy. The changes under stabilizer therapy suggest a possible pathophysiological role of ATTR-directed nAbs. Prospective evaluations and research on the clinical courses in relation to nAbs levels will provide further insights on their role on ATTR pathogenesis and their role in therapy monitoring. These data indicate a key role of nAbs in the pathophysiology, systemic tolerance and progression of ATTR-CM.

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Author contributions

CRedit: **Stephan Settelmeier**: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing; **Daniel Agranovski**: Conceptualization, Data curation, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing; **Maximilian Steinhardt**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing; **Sarah Waydhas**: Investigation, Visualization, Writing – original draft; **Ian Gering**: Formal analysis, Investigation, Methodology, Visualization, Writing – original draft; **Vladimir Cejka**: Data curation, Investigation, Writing – original draft; **Caroline Morbach**: Data curation, Formal analysis, Investigation, Writing – original draft; **Stefan Störk**: Data curation, Investigation, Writing – original draft; **Dieter Willbold**: Formal analysis, Investigation, Methodology, Project administration, Writing – original draft; **Ralf Küppers**: Data curation, Formal analysis, Methodology, Writing – original draft; **Julia Vogel**: Data curation, Formal analysis, Investigation, Writing – original draft; **Lars Michel**: Data curation, Formal analysis, Investigation, Methodology, Resources, Supervision, Writing – original draft; **Alexander Carpinteiro**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft; **Hans. Christian Reinhardt**: Data curation, Methodology, Supervision, Writing – original draft; **Richard Dodel**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing; **Tienush Rassaf**: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing; **Alexander J. Ross**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing; **Ulrike B. Hendgen-Cotta**: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation,

Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Disclosure statement

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