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Original article

Antibodies to calnexin and mutated calreticulin are common in human sera

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ABSTRACT

Purpose of the study: Calreticulin is an endoplasmic reticulum chaperone protein, which is involved in protein folding and in peptide loading of major histocompatibility complex class I molecules together with its homolog calnexin. Mutated calreticulin is associated with a group of hemopoietic disorders, especially myeloproliferative neoplasms. Currently only the cellular immune response to mutated calreticulin has been described, although preliminary findings have indicated that antibodies to mutated calreticulin are not specific for myeloproliferative disorders. These findings have prompted us to characterize the humoral immune response to mutated calreticulin and its chaperone homologue calnexin.
Patients and methods: We analyzed sera from myeloproliferative neoplasm patients, healthy donors and relapsing-remitting multiple sclerosis patients for the occurrence of autoantibodies to wild type and mutated calreticulin forms and to calnexin by enzyme-linked immunosorbent assay.
Results: Antibodies to mutated calreticulin and calnexin were present at similar levels in serum samples of myeloproliferative neoplasm and multiple sclerosis patients as well as healthy donors. Moreover, a high correlation between antibodies to mutated calreticulin and calnexin was seen for all patient and control groups. Epitope binding studies indicated that cross-reactive antibodies bound to a three-dimensional epitope encompassing a short linear sequence in the C-terminal of mutated calreticulin and calnexin.
Conclusion: Collectively, these findings indicate that calreticulin mutations may be common and not necessarily lead to onset of myeloproliferative neoplasm, possibly due to elimination of cells with mutations. This, in turn, may suggest that additional molecular changes may be required for development of myeloproliferative neoplasm.

Introduction

Molecular chaperones assist the conformational folding and assembly of macromolecular structures and have important functions in cell growth and homeostasis [1–5]. Calreticulin (CALR) and calnexin (CNX) are the most abundant representatives of a small family of lectin chaperone proteins, residing in the endoplasmic reticulum (ER). As typical chaperone proteins CALR and CNX are involved in quality control of (glyco)protein folding in addition to maintaining cellular Ca^{2+} homeostasis and cellular homeostasis in general [1–9]. Moreover, both chaperones are part of the peptide loading complex, which facilitates the loading of peptides onto major histocompatibility complex (MHC) class I molecules, implying an important role in immune surveillance [10–12].

The *CALR* gene on chromosome 19p13.2 consists of 9 exons and 8 introns and encodes a calcium-dependent protein of 417 residues, including a signal peptide of 17 amino acid residues [13,14]. The mature CALR protein contains an amino-terminal N-domain

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(residues 18–204) and a proline-rich P-domain with three sets of repeat sequences (residues 205–305). Moreover, CALR contains a carboxyterminal C-domain (residues 306–417), ending with a KDEL ER retention signal [13–15] (Fig. 1). The three-dimensional structure of CALR comprises a β-sandwich and a β-hairpin. The β-sandwich is formed by the N-domain and the first part of the C-domain, whereas the β-hairpin is formed by the P domain. Moreover, two α-helices are located in the last part of the C-domain. The first α-helix covers the lower sheet of the β-sandwich, whereas the second α-helix continues upwards to contact the P-domain [15,16].

The *CNX* gene on chromosome 5q35.3 encodes a protein with sequence and structural homology to CALR, although CNX has a longer P-domain, containing 4 sets of repeats. Moreover, the C-terminal part contains a short transmembrane region and a short cytoplasmic tail (Fig. 1) [17–19]. In contrast to CALR, which is a soluble luminal protein, CNX is anchored in the cellular membrane through a transmembrane helix. Moreover, CNX contains a C-terminal cytosolic RKPPRRE motif, which is essential for ER retention [19]. Although of similar structure to CALR, CNX is nearly 200 amino acids longer, which primarily is ascribed the presence of longer loop regions, an extended P-domain and an acidic transmembrane domain (Fig. 1).

CALR and CNX have been reported to be associated with several diseases such as various cancer types, some neurological diseases, chronic myeloproliferative neoplasms (MPNs), and some autoimmune diseases [20–26].

Cancers are characterized by one or more of several hallmarks, including sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism and evading immune destruction [27,28]. In cancers, CALR and CNX expression levels are generally increased due to cell growth and ER stress, induced by several chemicals, or physicochemical stress under necrotic or stressful conditions. In addition, CALR may translocate to the cell surface, where it can function as an opsonin to induce immunogenic cell death [21,24-26].

MPNs are caused by clonal expansion of myeloid lineage precursor cells and include polycythemia vera (PV), essential thrombocythemia (ET) and myelofibrosis (MF) [29–32]. Clinical symptoms overlap between these diseases, but typically include increased production of red blood cells in PV, elevated platelets in ET and fibrosis of the bone marrow in MF [29–32]. In MPNs, characteristic mutations of the *Janus kinase (JAK) 2* gene and the *myeloproliferative leukemia virus (MPL)* oncogene encoding the thrombopoietin receptor (TPOR) are responsible for the majority of cases. In contrast, alterations of the *CALR* gene (insertions and deletions (INDELS)) are seen in approximately 25 - 35% of patients with ET and primary MF (PMF) [31–34]. INDELS have been identified in exon 9 and primarily result in frameshift mutations, yielding a polybasic sequence with a common C-terminal peptide sequence. This C-terminal modification is in great contrast to the acidic C-terminal found in the CALR wild type (wt) [32–36]. The two most common mutations are a 5-base pair insertion (K385fs*47) and a 52-base pair deletion (L367fs*46) also termed type 1 and type 2 mutations, respectively [36]. Type 1 mutations eliminate all of the negatively charged amino acids in the C terminus of CALR, whereas type 2 mutations eliminate approximately 50% or the negatively charged amino acids [37]. Mutations in *JAK2*, *CALR* or *MPL* genes are commonly referred to as driver mutations, a term used to describe changes in the DNA sequences of genes that cause cells to transform into cancer cells.

In addition to MPN, CALR and CNX have been proposed to be associated with neurological diseases. These neurological diseases encompass a diverse spectrum of diseases, e.g., autoimmune diseases such as multiple sclerosis (MS) and other neurologic disorders, some of which are considered protein folding diseases [38,39]. Although CALR and CNX occasionally have been reported to play a role in these diseases, no consistent pattern has emerged [20,40-42].

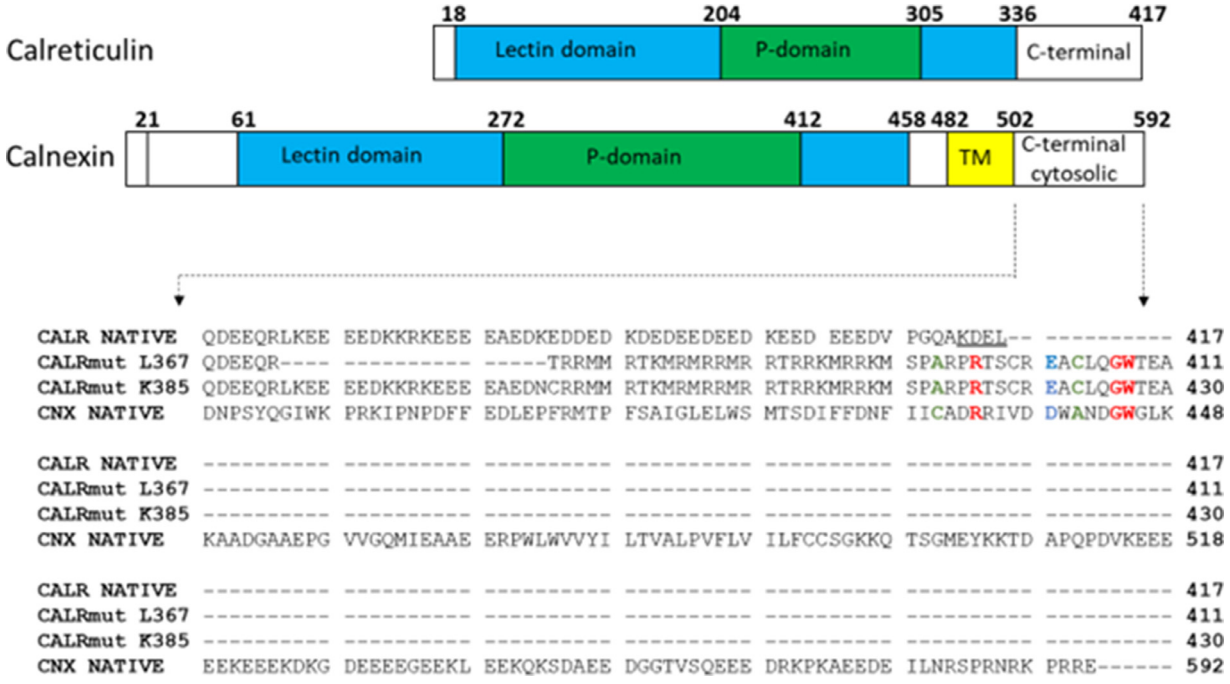

Fig. 1. Schematic presentation of calnexin (CNX) and calreticulin (CALR) forms and location of C-terminal CALR frameshift mutations (CALRmut). CALR and CNX contain a lectin domain, a P-domain and an acidic C-terminal. In addition, CNX contains a transmembrane (TM) helix in the C-terminal and a C-terminal exposed in the cytosol. The different CALR regions are coloured according to Boelt et al. [16], and the C-terminal part from residue 361 of native CALR, CALRmut L367, and CALRmut K385 are presented. The KDEL sequence of native CALR is underlined. CNX is colored according to Koslov et al. [19] and residues 482 – 502 are presented, containing the TM domain and the C-terminal. Seven amino acids between CALRmut and CNX have common chemical and/or structural similarities. Red colors represent identical amino acids, blue colors represent amino acids sharing chemical properties, green colors represent similar chemical properties.

Finally, hematological disturbances are common in many autoimmune diseases, in particular the systemic autoimmune diseases [43–45]. Autoantibodies are a common feature of autoimmune diseases, but their frequencies and pathophysiological roles differ much between various diseases, ranging from essentially universal to sporadic occurrences [46–52]. Posttranslational modifications have been suggested to be a major cause of autoantibody generation [53–55], although posttranslational modifications associated with autoimmune diseases are relatively few. In addition, mutations and other genetic alterations generating neoepitopes may also contribute to generation of autoantibodies.

Since mutations may generate autoantigens evading self-tolerance and since autoantibodies to CALR and other chaperones have been reported [56–67], we investigated the occurrence of antibodies to mutated CALR (CALRmut) in MPN patients, MS patients and healthy individuals and compared their reactivities with CALR wt IgG and CNX IgG levels.

Materials and methods

Materials

Alkaline Phosphatase (AP)-conjugated goat-anti human IgG, α-Cyano-4-hydroxycinnamic acid (ACCA), biotin, *p*-nitrophenylphosphate (*p*NPP) substrate tablets, streptavidin, trifluoroacetic acid (TFA), triisopropylsilane (TIS), Tentagel S RAM resin, N,N-diisopropylethylamine (DIEA), thioanisole (THIO) and 9-fluorenylmethoxycarbonyl (Fmoc)-L-amino acids were from Sigma Aldrich (St. Louis, MO, USA). PolySorp microtiter plates (96-wells) were from Thermo Fisher Scientific (Hvidovre, Denmark). AP-substrate buffer (1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8), carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and Tris-Tween-NaCl (TTN) buffer (0.05 M Tris, 0.3 M NaCl, 1% Tween 20, pH 7.5) were from SSI Diagnostica (Hillerød, Denmark). Recombinant CALR wt, CALRmut K385, CALRmut L367 and CNX were from Baltymas (Vilnius, Lithuania). The proteins were produced in yeast and characterised as previously described [68,69]. 1-hydroxy-7-azabenzotriazole (HOAt) was from ChemPep (Wellington, USA). Piperidine (PIP), dimethylformamid (DMF), diethylether, ethanol, acetonitrile and dichloromethane (DCM) was from VWR Chemicals (Gliwice, Poland). Hexafluorophosphate azabenzotriazole tetramethyl uranium (HATU) was from Iris Biotech GMBH (Markredwitz, Germany).

Patient samples and human ethics

MPN patient samples (n = 50) were collected at the Department of Hematology, Zealand University Hospital Roskilde, Roskilde, Denmark and were non-selected patients being referred to the department (Appendix 1A). Of these patients, 18 (36%) had mutations in the *JAK2* gene, 27 (54%) were positive for mutations in *CALR*, 2 had an *MPL* mutation, whereas 3 did not have mutations in any of the three genes (triple negative). Of the patients with mutations in *CALR*, 14 (52%) and 7 (26%) had type 1 or type 2 mutations, respectively, whereas the remaining 22% had other mutations or were not typed (Appendix 1). The clinical diagnoses for MPN patients with *CALR* mutations were mainly MF or PMF (81%). In contrast, the clinical diagnoses were mainly PV for patients with *JAK* mutations (61%) (Table 1).

MS patient samples (n = 29) were collected at the Department of Neurology, Rigshospitalet Glostrup, Copenhagen, Denmark and fulfilled the most recent McDonald Criteria [70]. All MS patients were diagnosed with replapsing-remitting MS (RRMS). Samples from healthy donors (HD), consisting of healthy blood donors (n = 12) were obtained from the Department of Clinical Immunology, Rigshospitalet, Copenhagen, Denmark. Healthy controls (HCs), which consisted of healthy blood donors and volunteers, were from Rigshospitalet and Statens Serum Institut (Copenhagen, Denmark) (n = 31). Furthermore, three apparently healthy persons had been found to have a mutation in *CALR* in a population screening program [71]. CNX and CALRmut antibody titers were determined for the three healthy persons, however, they were not included in the data and the statistical analyses due to the small sample size (Appendix 1B). Table 2 and Appendix 1 shows details of the samples used in this study.

Table 1
Disease characteristics of myeloproliferative patients categorized according to driver mutation and disease.

Mutation/diagnosis	PV	ET	PMF	preMF	n (%)
CALR	–	5	16	6	27 (54)
JAK2	11	4	3	–	18 (36)
MPL	–	1	1	–	2 (4)
Negative	1	2	–	–	3 (6)

CALR, Calreticulin; ET, Essential thrombocythemia; JAK2, Janus kinase; MF, myelofibrosis; MPL, Myeloproliferative leukemia virus oncogene: PMF, Primary myelofibrosis: PV, Polycythemia vera.

Investigations were carried out according to current guidelines for experiments involving human material following the ethical recommendations of the statement of Helsinki. Consent was obtained from all participants and from the regional scientific committee of Copenhagen (project no H-19,036,891) and the regional scientific ethics board of Zealand (project no SJ-456). A flowchart representing samples and antigens tested in this study is depicted in Fig. 2.

Synthetic peptides

Biotinylated peptides covering the C-terminal of CALRmut (Biotin-ARPRTSCREACLQGWTEA) with sequence similarity to CNX (Biotin-CADRRIVDDWANDGWGLK) were synthetized using traditional Fmoc-based solid-phase peptide synthesis as previously described [72]. Briefly, peptides were synthesized manually in syringes equipped with PTFE filters using Tentagel S RAM resin (50 mg, loading: 0.24 mmol/g). Fmoc-protected L-amino acids and coupling reagents (HOAt, HATU and DIEA) were used in fourfold excess relative to resin loading. Coupling was performed for 2 h (h) at room temperature (RT). After each coupling resins were rinsed with DMF. Fmoc was removed using 20% v/v PIP in DMF for 20 min. Peptides and side-chain protecting groups were cleaved from the resin using a cocktail of 95% TFA, 2.5% THIO and 2.5% TIS for 2 h at RT. Biotin was coupled to the N-terminal of the final peptide using the same procedure as described above. Following coupling of biotin, resins were rinsed with DMF, DCM and ethanol and freeze-dried overnight (ON). Finally, TFA was removed by evaporation and free peptides were precipitated in ether, redissolved in 10% aqueous acetic acid and lyophilized. Molecular weights of the peptides were identified and

Table 2
Patient and control material tested for reactivity in the current study.

Material	N	Age (range)	F/M ratio	Characteristics
MPN	50	57 (33 – 79)*	64/36	Myeloproliferative neoplasm patients (Table 1, Appendix 1)
HCMC	3	NK	1/2	Healthy calreticulin mutation carriers
RRMS	29	42 (23–58)*	19/10	Relapsing-remitting multiple sclerosis patients
HD	12	NK	NK	Healthy blood donors
HC	31	42 (32–52)	24/7	Healthy volunteers
DP	100	NK (18–60)	NK	Healthy blood donors

DP: donor pool, HCMC, healthy calreticulin mutation carrier; HD, healthy donor; HC, healthy controls (volunteer); MPN, myeloproliferative neoplasm; NK, not known; RRMS, relapsing-remitting multiple sclerosis. *Age at diagnosis.

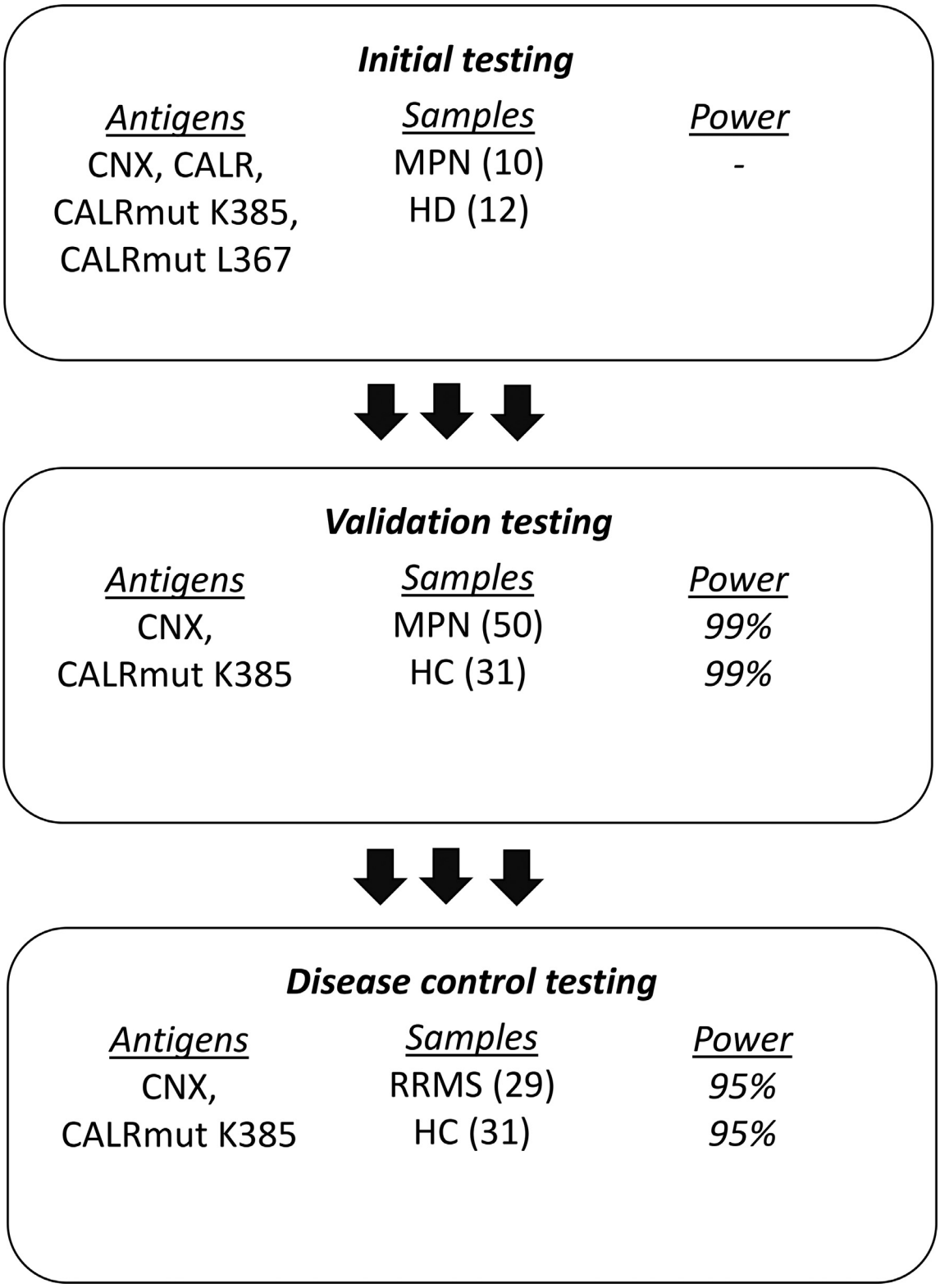

Fig. 2. Flowchart illustrating workflow for testing of samples originating from myeloproliferative neoplasm (MPN) patients, healthy donors (HD), healthy controls (HC) and relapsing-remitting multiple sclerosis (RRMS) patients.

confirmed using MALDI-TOF-MS, where the free peptides were dissolved in acetonitrile (50%) and TFA (0.1%) to a final concentration of 1 mg/mL. 1 μL of the sample was mixed with ACCA matrix whereafter the molecular weight was determined. Peptide purity was determined by HPLC and purities above 80% were accepted.

Enzyme-linked immunosorbent assay

96-well PolySorp microtiter plates were coated with CALR wt, CNX, CALRmut K385 (CALR type-2 mutation) or CALRmut L367 (type-1 mutation) (1 μg/mL) diluted in carbonate buffer ON at 5 °C.

Next, the wells were rinsed with TTN (3 × 5 min) and blocked with TTN for 1 h at RT on a shaking table. Subsequently, the wells incubated with patient sera (1:100 diluted in TTN) for 1 h at RT on a shaking table, followed by washing with TTN (3 × 5 min). AP-conjugated goat-anti human IgG (1:2000 dilution in TTN) or AP-conjugated goat-anti mouse IgG (1:1000 dilution in TTN) were added to all wells and incubated for 1 h. Following rinsing with TTN (3 × 5 min), *p*NPP diluted in AP-substrate buffer (1 mg/mL) was added to all wells. Bound antibodies were quantified by measuring the absorbance at 405−650 nm after sufficient color reaction using a microtiter plate reader (Molecular Devices, Menlo Park, CA, USA). Wells incubated with TTN were used for measurement of background. A standard curve was created using a donor pool, which was included on all plates using 2-fold dilutions (1:100, 1:200, 1:400, 1:800, 1:1600 and 1:3200).

For test of MPN patient serum reactivity to biotinylated CALR-mut and CNX peptides, PolySorp microtiter plates were precoated with streptavidin (1 μg/mL/well) in carbonate buffer ON. Following precoating, wells were rinsed with TTN and blocked with TTN for 1 h at RT, whereafter plates were coated with biotinylated peptides (1 μg/mL) for 1 h at RT on a shaking table. Next plates were washed with TTN and MPN samples were added to each well (1:100 dilution in TTN). Bound antibodies were quantified as described above.

Competitive inhibition assay

PolySorp microtiter plates were coated with CALR wt, CALRmut K385 and CNX as described above. Following coating, wells were rinsed and blocked as previously described. A MPN pool consisting of sera from MPN patients (n = 4) with the highest antibody reactivity (final concentration 1:100) was mixed with either CALR wt, CNX or CALRmut K385 in the following protein concentrations: 0.1 μg/mL, 1 μg/mL, 10 μg/mL and 100 μg/mL and preincubated for 1 h at RT on a shaking table. Following blocking of microtiter wells and preincubation of samples, the wells incubated with the various antigen/sera/antibody mixtures for 1 h at RT on a shaking table. Subsequently, the plates were washed with TTN (3 × 5 min), and AP-goat-anti human IgG (1:2000 dilution in TTN) was added to all wells and incubated for 1 h at RT on a shaking table. Bound antibodies were quantified as described above using AP-substrate buffer.

Data analysis

MyAssays.com was used to generate a 4-parameter logistic curve fit for calculation of IgG concentrations (U/ml). Statistical analyses were generated using Prism 5 (Graphpad, San Diego, CA, USA). Data are presented as mean ± standard deviation of mean. Statistical significance was assessed by nonparametric unpaired two-tailed Mann Whitney test. Significant differences are indicated by *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. For correlation analysis of results the Pearson's r, p-value and 95% confidence interval were determined for each analysis. Correlations were classified accordingly: r: 0.0−0.19 very low, 0.2−0.39 low, 0.4−0.59 moderate, 0.6−0.79 high, 0.8−1.0 strong correlation.

Power calculations were performed using GraphPad StatMate using the sample sizes, standard deviations and a significance level of 0.05 (two tailed).

Results

Determination of specific and non-specific antibody binding to calnexin and calreticulin forms in myeloproliferative neoplasm patients and healthy donors

Initially, randomly picked MPN patient (n = 10) and HD (n = 12) serum samples were analysed for reactivity to CNX and CALRmut K385 in ELISA. As solid phase immunoassays with human samples may exhibit issues with non-specific binding [73], specific and non-specific antibody binding for a number of samples was evaluated using coated and non-coated wells (Fig. 3).

For all sera, the background reactivity (- coat) was lower when compared to specific binding (+ coat). Moreover, the background level for the individual samples was similar to the negative assay control (TTN), indicating that the reactivity to CNX and CALRmut K385 resulted from specific binding. The antibody titers to the two antigens varied between the individual samples, nevertheless CNX and CALRmut K385 IgG were detected in essentially all samples.

Next, antibody levels to CNX, CALR wt, CALRmut K385 and CALRmut L367 were evaluated for the MPN and HD samples initially tested. IgG levels to the four proteins generally appeared to be of the order CALRmut K385 > CNX > CALRmut L367 > CALR wt with a few individual variations (Fig. 4). Thus, the highest antibody level was observed to CALRmut K385 for MPN patients (mean: 1.2 A.U.), in comparison to CNX (mean: 0.73 A.U.), CALRmut L367 (mean: 0.52 A.

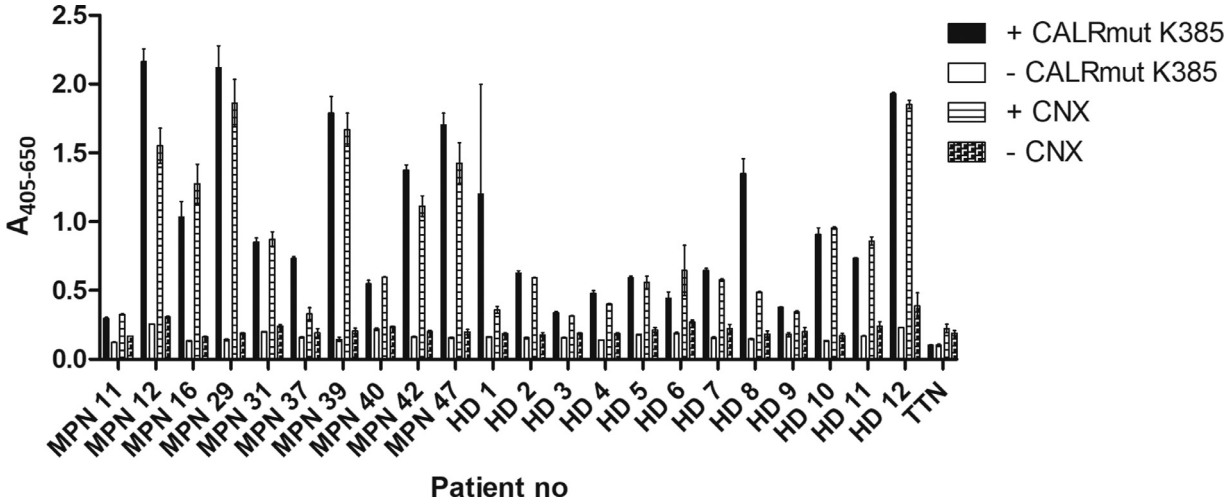

Fig. 3. Specific binding of antibodies in myeloproliferative neoplasm (MPN) patient and healthy donor (HD) samples to calnexin (CNX) and calreticulin (CALR) K385 analysed by enzyme-linked immunosorbent assay. Sera from 10 MPN patients and 12 HDs were tested for binding with (+) and without antigen coating (-). Tris-Tween-NaCl (TTN) was used as background control.

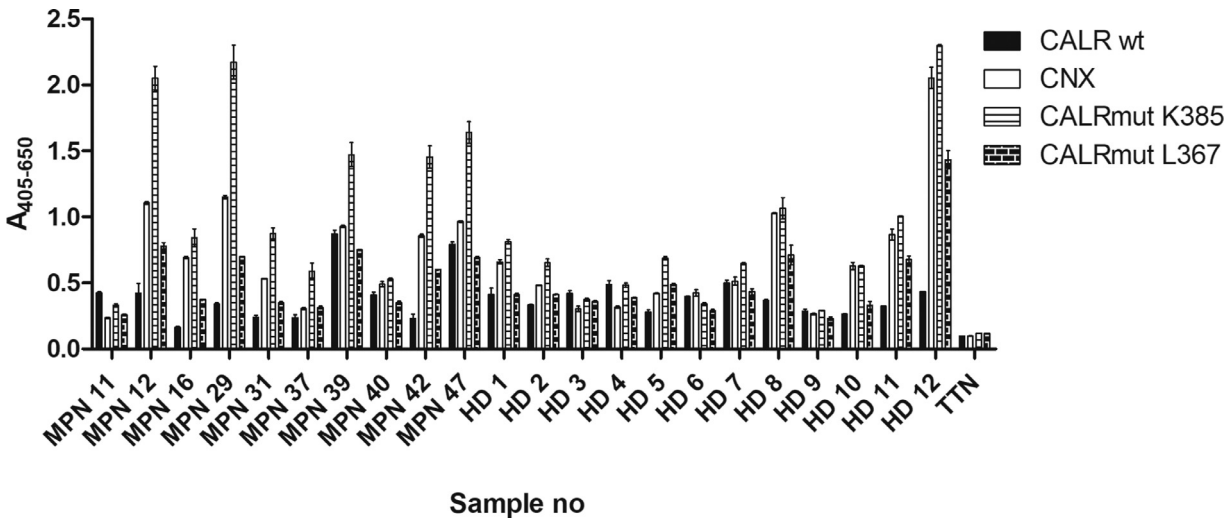

Fig. 4. Reactivity of myeloproliferative neoplasms (MPN) patient samples and healthy donor (HD) samples to calnexin (CNX) and calreticulin (CALR) forms analysed by enzyme-linked immunosorbent assay. Sera from 10 MPN patients and 12 HD samples were tested for reactivity to CALR wild type (wt), CNX, CALRmut K385 and CALRmut L367. Tris-Tween-NaCl (TTN) was used as background control.

U.) and CALR wt (mean: 0.41 A.U.). More than 50% MPN patient samples tested showed elevated CNX and CALRmut K385 IgG levels compared to CALR wt and CALRmut L367 IgG. In addition, the majority of HD samples reacted weakly to the antigens tested, whereas a few sera showed elevated IgG reactivity to CALRmut K385 and CNX as well.

Test of correlation between antibody titers to calnexin and calreticulin forms

Initial screenings demonstrated elevated IgG reactivities to CNX, CALRmut K385 and CALRmut L367 proteins in comparison to CALR wt. To analyze this further, correlation studies were conducted to examine, whether the measured antibody levels were associated. Fig. 5 illustrates correlation analyses between the four IgG groups: CNX, CALR wt, CALRmut K385 and CALRmut L367.

As presented, weak positive correlations were observed for CNX IgG and CALR wt IgG (r = 0.3492, p = 0.3226) and CALR wt IgG and CALRmut K385 IgG (r = 0.3066, p = 0.389) (Table 3). Moreover, a moderate positive correlation was determined between CALR wt IgG and CALRmut L367 IgG (r = 0.554, p = 0.0956), although not statistically significant. In contrast, strong positive correlations were determined between antibody levels to CNX and CALRmut K385 (r = 0.9696, p < 0.0001), CNX and CALRmut L367 (r = 0.9430, p < 0.0001) and CALRmut L367 and K385 (r = 0.9393, p < 0.0001). Only CNX IgG showed strong positive correlations to the two CALRmut IgGs, indicating that the C-terminal of CALRmut may contain structural or sequence similarities to CNX, which are absent in CALR wt. This was confirmed when examining IgG correlations between the two CALRmut and between CALR wt to the remaining proteins. Here it was found that, correlations with CALR wt IgG to the remaining IgG levels were weak, whereas the correlations between CALRmut; L367 and K385 were very strong, similar to CNX IgG correlations with CALRmut L367 IgG and K385 IgG.

Determination of antibody binding to calnexin and calreticulin mutation K385 in an expanded myeloproliferative neoplasm cohort and control cohorts

Based on preliminary screenings, a cohort of 50 MPN samples (n = 50) and HCs (n = 31) were tested for reactivity to CNX and CALRmut K385 by ELISA (Fig. 6).

As presented in Fig. 6A, no significant difference in IgG levels to CNX and CALRmut K385 in MPN and HC samples was determined (p = 0.4849 for CNX and p = 0.9773 for CALRmut K385). Similarly, no significant difference in CNX IgG and CALRmut K385 IgG was observed neither for MPN (p = 0.4495) nor HCs (p = 0.8609), supporting preliminary findings. Moreover, strong positive correlations were observed between CNX IgG and CALRmut K385 IgG in MPN patient samples (r = 0.8080) and HC samples (r = 0.9367) (Fig 6B-C).

Due to the occurrence of antibodies to CNX and CALRmut in both MPN patients and HCs, sera from RRMS patients (n = 29) were tested for reactivity to CNX and CALRmut K385 as well.

As presented, CNX IgG titers were slightly elevated when compared to CALRmut K385 although not significant (p = 0.0872) (Fig. 6A). In fact, a strong and statistically significant correlation was found between CNX and CALRmut K385 IgG in RRMS patient samples (r = 0.9281, p < 0.0001) (Fig. 6E). No difference in IgG titers was found when comparing antibody levels for RRMS and HCs cohorts (p = 0.3074 for CNX and p = 0.8475 for CALRmut K385, respectively).

Collectively, the current findings indicate that antibodies to CNX and CALRmut K385 are not specific for MPN disease, as elevated antibody levels were determined in healthy individuals and RRMS samples as well. The latter was confirmed when comparing all CNX IgG and CALRmut IgG titers for MPN, HC and RRMS sera samples together, as a strong correlation for IgG levels was determined (Fig. 6F) (r = 0.8288, p < 0.0001).

Identification of binding sites of antibodies from myeloproliferative neoplasm sera to mutated calreticulin and calnexin

Strong correlations and the widespread occurrence of antibodies to CNX and CALRmut suggested that cross-reactivity between the antigens may exist. Based on this, we investigated the ability of CNX, CALR wt and CALRmut K385 to inhibit IgG cross-reactivity in a competitive inhibition assay.

As depicted in Fig. 7A, CNX and CALRmut K385 proteins inhibited IgG binding in a concentration-dependent manner, suggesting that antibodies to CNX and CALRmut K385 are cross-reactive and recognize an epitope present in both CNX and CALRmut. In contrast, CALR wt (Fig. 7B) did not inhibit antibody binding to the same extent, conforming to earlier findings.

In an attempt to identify the cross-reactive binding sites, C-terminal biotinylated CALRmut K385 (Biotin-ARPRTSCREACLQGWTEA)

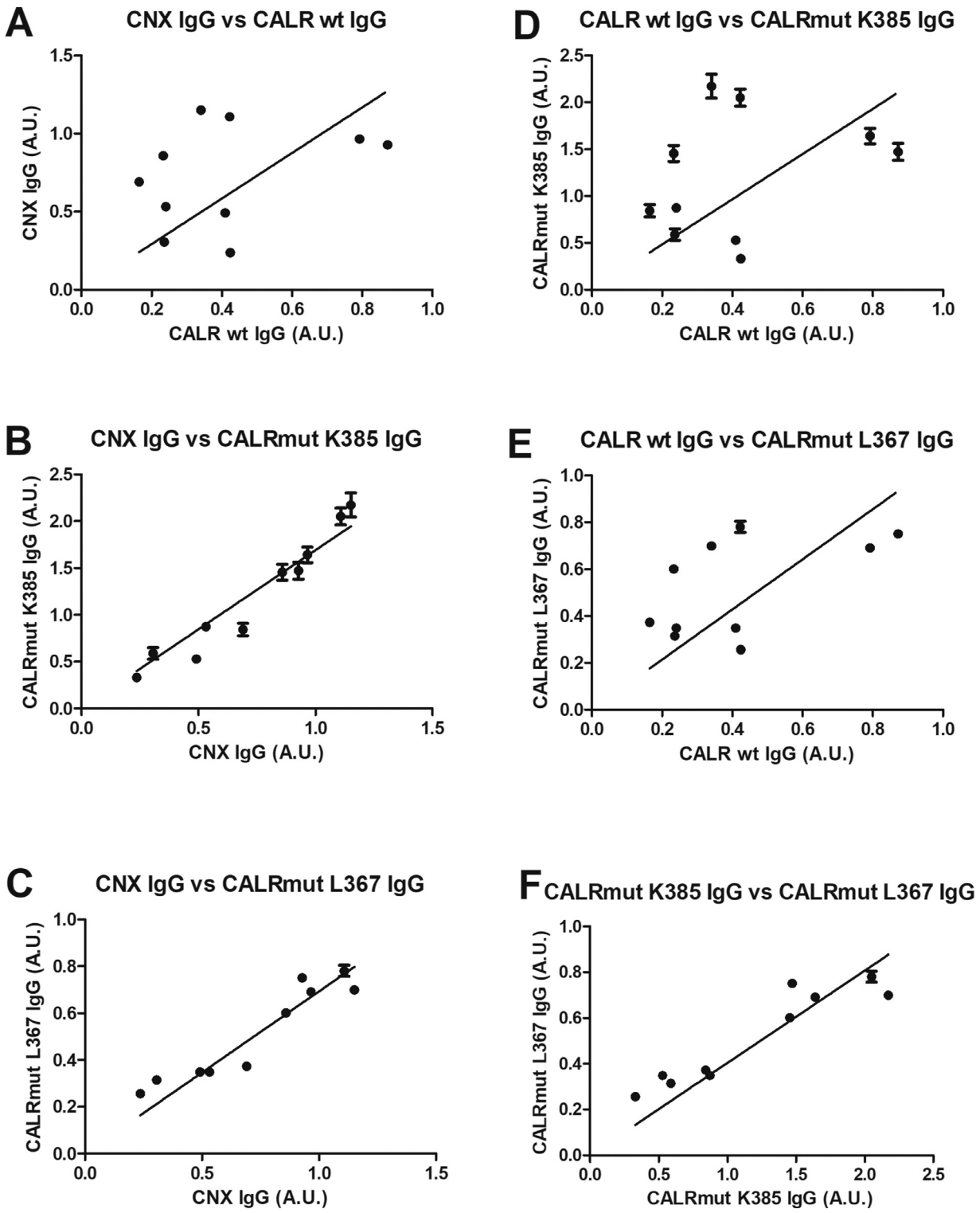

Fig. 5. Correlation analyses of antibodies from myeloproliferative neoplasms (MPN) samples to calnexin (CNX), mutated calreticulin (CALRmut) K385 and L367 and calreticulin (CALR) wild type (wt). A. CNX vs CALR wt. B. CNX vs CALRmut K385. C. CNX vs CALRmut L367. D. CALR wt vs CALRmut K385. E. CALR wt vs CALRmut L367. F. CALRmut K385 vs CALR L367.

and CNX (Biotin-CADRRIVDDWANDGWGLK) peptides were synthesized and tested for reactivity using an MPN pool by ELISA. However, no reactivity was found to the biotinylated peptides (results not shown). Collectively, current findings indicated that IgG cross-reactivity to CNX may be ascribed to conformational regions in the C-terminal of CALRmut K385.

Correlation between antibody titers and driver mutations

Following determination of CALRmut K385 and CNX IgG titers, possible correlations between driver mutations and antibody levels were analysed. For this analysis, patient samples were classified based on their driver mutation and an IgG titer above 20 U/mL. As

Table 3
Correlation analyses between antibody levels to calnexin (CNX) and calreticulin (CALR) forms.

IgG correlation	Pearson r	95% confidence interval	P value
CNX vs CALR wt	0.3492	−0.3596 – 0.8025	0.3226
CNX vs CALR K385	0.9696	0.8729 – 0.9930	< 0.0001
CNX vs CALR L367	0.9430	0.7712 – 0.9868	< 0.0001
CALR wt vs CALRmut K385	0.3066	−0.4005 – 0.7848	0.3890
CALR wt vs CALRmut L367	0.5554	−0.1143 – 0.8780	0.0956
CALRmut K385 vs CALRmut L367	0.9393	0.7577 – 0.9859	< 0.0001

presented in Table 4, the majority of MPN patients (54%) presented with mutations in the *CALR* gene, whereas mutations in the *JAK2* and *MPL* gene were found in 36 and 4% of MPN cases, respectively. Six% were negative for driver mutations. Approximately 74% of MPN patients presenting with *CALR* mutations were positive for antibodies to both CNX and CALRmut K385, whereas 67 and 56% of MPN patients positive for *JAK2* mutations expressed elevated CNX and CALRmut K385 IgG titers, respectively. In contrast, the percentage of patients positive for CNX IgG and CALRmut K385 IgG were reduced in the *MPL* group and in the group negative for all three driver mutations. In fact, none of the patients positive for *MPL* mutations expressed CALRmut K385 IgG, whereas 50% expressed antibodies to CNX. Only one out of three MPN patients negative for driver mutations expressed antibodies to CNX and CALRmut K385. No difference in CNX and CALRmut K385 IgG titers in samples from patients positive for *CALR* and *JAK2* mutations was observed, moreover no difference in IgG levels between male and female contributors was determined (results not shown).

As presented in Table 1, mutations in *CALR* were mainly associated with MF and to a lesser extent ET. No difference in CNX and CALRmut K385 IgG was found between the individual disease categories and no differences were determined between male and female patients in the respective disease groups (results not shown).

Discussion

This work presents the first study, describing specific and inhibitable autoantibody reactivity to mutated CALR and CNX in MPN and HCs. Moreover, strong and highly significant correlations were found between autoantibodies to CALRmut and CNX, which cross-reacted between mutated CALR forms and CNX. In contrast, very low autoantibody levels were found to CALR wt, which did not correlate with CNX IgG nor CALRmut IgG. Our findings are in accordance with early findings, describing the occurrence of autoantibodies to CALR in various cancers and systemic autoimmune diseases [56–66], although, the affinity and specificity of these have been questioned [74].

Intriguingly, the autoantibodies to mutated CALR and CNX were found in all groups investigated (Fig. 6). These results raise the important question, whether mutated CALR are the cause of these autoantibodies cross-reacting with CNX or vice versa. Based on the knowledge that genetic mutations may result in generation of immune stimulating neoepitopes, our findings would argue that CALRmut forms are the causative neoautoantigens, indicating a primary role for the common C-terminus in CALRmut forms. This would explain the lack of cross-reactivity to CALR wt, since the C-terminus of CALR has no homology to the C-terminus of CALRmut. This is in accordance with the fact CALR wt is an abundant ubiquitous cellular protein, which is tolerated by the immune system.

Results presented in this study may suggest that the occurrence of somatic mutations in *CALR* is a common phenomenon, which normally do not lead to MPN disease. These findings remain to be confirmed and elaborated thoroughly, however an explanation may be that the mutated cells are neutralized through immune surveillance and "altered cell" killing. During the process of mutated cell killing and subsequent removal of cellular debris, CALRmut forms become exposed, ultimately resulting in the generation of autoantibodies. This theory is supported by studies demonstrating CALR mutations in healthy carriers [71] and that healthy individuals as well as MPN patients have T (memory) cell responses to mutated CALR [75,76]. The presence of T memory cell responses supports the theory that healthy individuals occasionally acquire CALR mutations, which usually are cleared by the immune system due to their immunogenicity, whereby T memory cells are generated [76]. These findings are supported by Cordua et al., describing that healthy individuals with low-allele burden of CALR mutations have circulating CALR-mutated cells in peripheral blood and show T cell responses to mutated CALR [71,77]. Collectively, our findings combined with existing literature demonstrate humoral as well as cellular immune responses to mutated CALR [71,75-77].

Results described within this study addresses potential differences in the physiological properties and roles of CALRmut and CALR wt. CALR is an ER chaperon protein, which participates in several immune-related processes [6–12]. Moreover, CALR is able to translocate to the cell surface in connection with apoptosis and immunogenic cell death and to induce phagocytosis of dying cells [78–81]. The complex process of immunogenic cell death is not entirely understood and the possible role of CALRmut in this process remains to be thoroughly examined, nevertheless CALRmut released from dying cells has been reported to suppress immune responses [82,83].

CALR and CALRmut have similar thermodynamic stabilities and the overall structures are therefore expected to be similar. Both CALR and CALRmut form dimers and oligomers, especially under physicochemical stress [69,84-87]. In fact, CALRmut dimers are thought to be responsible for some of the transforming properties of CALRmut forms, as the dimers are capable of stimulating the thrombopoietin receptor [86,87]. Due to the overall structural and conformational similarities of CALR forms, the differences in the physiological and (auto)immunological properties may be related to the different C-terminal amino acid sequences. In contrast to CALR wt, which contain a poly-acidic amino acid sequence, ending in a KDEL ER retrieval sequence, CALRmut forms have a polybasic sequence of varying length, ending in a common C-terminal sequence (Fig.1) [15,32-36]. Moreover, the C-terminus of CALRmut forms contain several cysteines, which may be directly related to the properties of CALRmut, possibly by interacting with the exposed disulfide bridge of CALR [67].

The differences in the C-terminus of CALRmut and CALR wt may also explain antibody cross-reactivity between CALRmut and CNX but not to CALR wt. CNX and CALR are homologous proteins with 45% sequence identity and similar overall structures (Fig.1) [15]. Homology between the proteins is restricted to the globular part of the proteins, which share an unusual β-sandwich composed of the N-terminal domain and part of the C-terminal domain, which contributes two strands to the sandwich. From the globular core extends a proline-rich β-hairpin (P-domain), which is somewhat longer in CNX [15]. Thus, epitopes involving the globular part of CNX, primarily containing structural and sequence homology to CALR, would be expected to be located to CALR as well. However, this was not confirmed, which may be explained by the absence of the mutated C-terminal in CALR wt as already mentioned (Fig. 1). Together, these structural similarities and differences suggest that CALRmut may have homology or similarity to part of the C-terminal of CNX. Actually, (Fig. 1) a short stretch of amino acids with a limited homology encompassing three identical amino acids (R, G, W), two conservative substitutions (D-E, D-Q (CNX-CALRmut)) and two A-C substitutions is found in the two proteins (C-A, A-C (CNX-CALRmut)). Nevertheless, synthetic peptides covering this stretch of amino acids did not react with MPN sera. Thus, a three-dimensional epitope involving the C-terminus together with adjacent parts of the globular region is

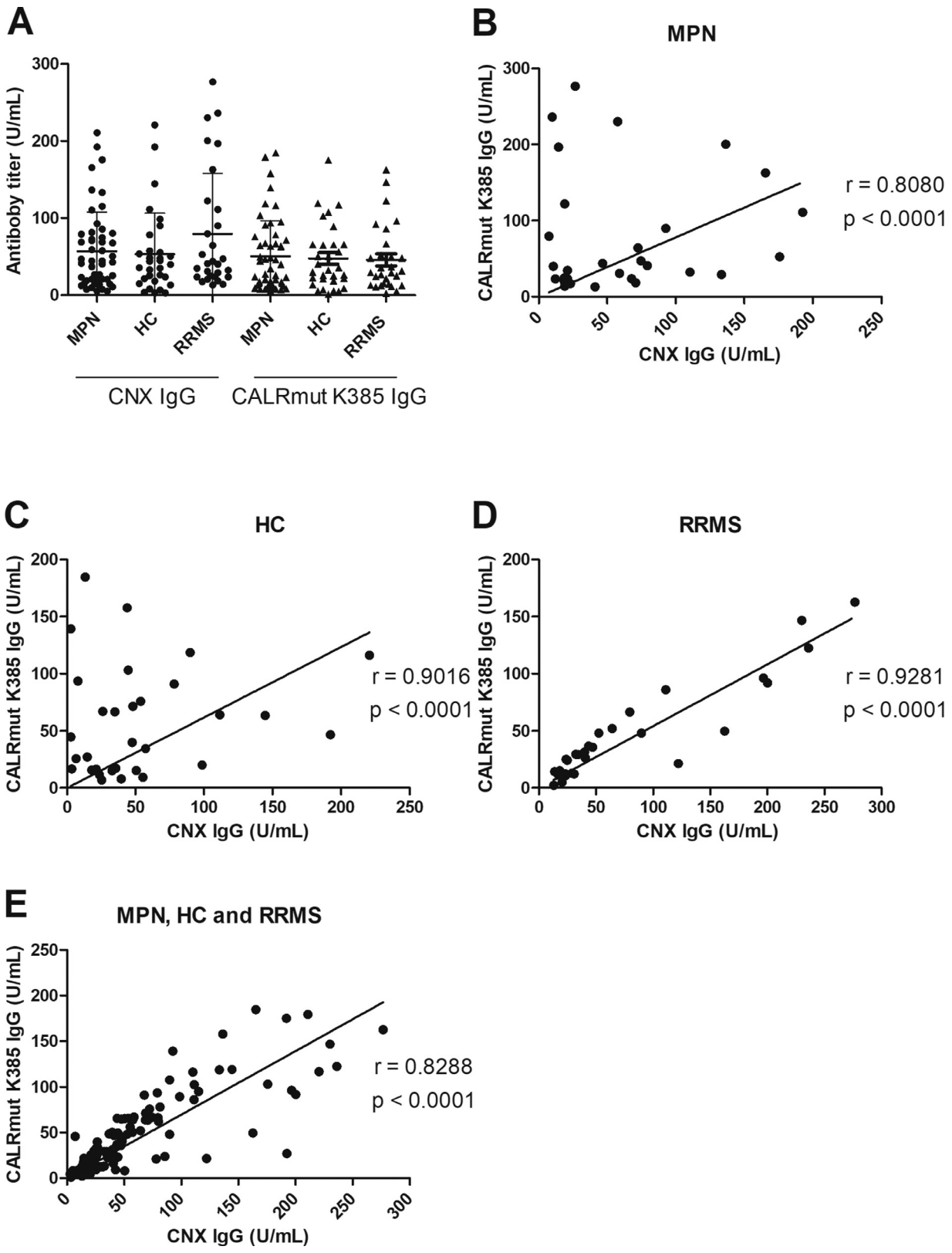

Fig.. 6. Antibodies to calnexin (CNX) and mutated calreticulin (CALRmut) K385 in myeloproliferative neoplasms (MPN), healthy control (HC) samples and relapsing-remitting multiple sclerosis samples (RRMS) analysed by enzyme-linked immunosorbent assay. A. Samples from 50 MPN patients, 31 HCs and 29 RRMS patients were tested for IgG reactivity to CNX and CALRmut K385. **B**. Correlation between CNX and CALRmut K385 IgG in MPN patients. **C**. Correlation between CNX and CALRmut K385 IgG in HC samples. **D**. Correlation between CNX and CALRmut K385 IgG in RRMS samples. **E**. Total IgG correlations between CNX and CALRmut K385 IgG in MPN, HC and RRMS samples.

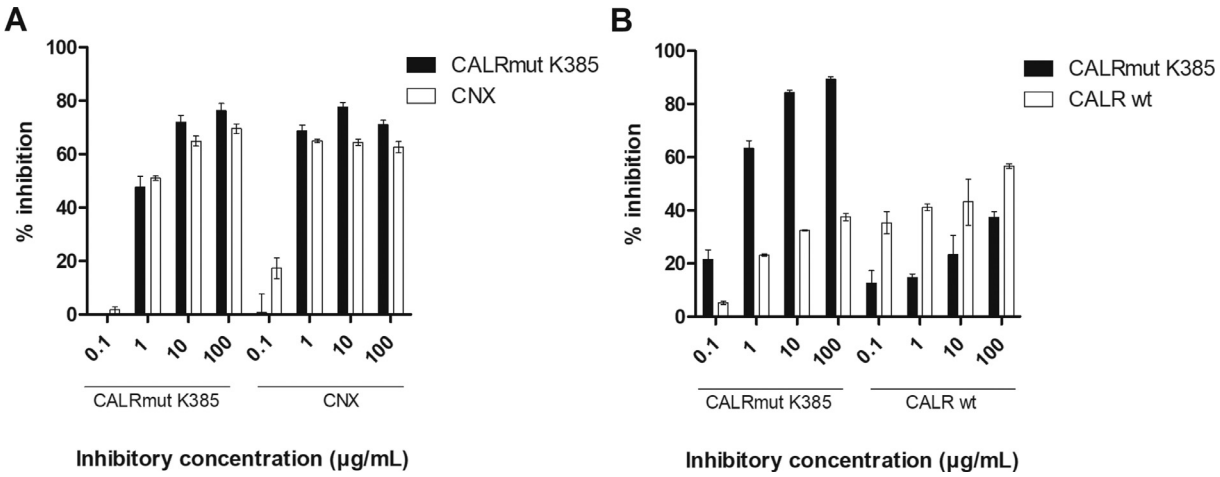

Fig. 7. Antigen inhibitory effects of CNX, CALR wt and CALRmut K385 analysed by competitive inhibition assay. A. Wells were coated with CALRmut K385 or CNX, whereafter the inhibitory effect of CALRmut K385 and CNX was determined. The inhibitory effect was determined relative to a control, where no inhibitor was added. **B**. Wells were coated with CALRmut K385 or CALR wt, whereafter the inhibitory effect of CALRmut K385 and CALR wt was determined. The inhibitory effect was determined relative to a control, where no inhibitor was added.

Table 4
Driver mutations in myeloproliferative (MPN) patients relative to IgG titers to calnexin (CNX) and mutated calreticulin (CALRmut) K385. In total 50 MPN patients were categorized relative to driver mutations and IgG titers. A cut-off of 20 U/mL was introduced.

Mutation gene	n (%)	CNX IgG	CALRmut K385 IgG
CALR	27 (54)	74%	74%
JAK2	18 (36)	67%	56%
MPL	2 (4)	50%	0%
Negative	3 (6)	33%	33%

believed to be the most promising candidate for a three-dimensional epitope. Such an epitope would presumably be sensitive to differences in the length of the polybasic stretch of mutated CALR forms, explaining most reactivity to CALRmut K385 compared to CALRmut L367, whether due to differences in steric hindrance or differences in intactness of the C-terminus [67]. Collectively, the exact nature of the epitopes of CALRmut forms and CNX remains to be elaborated, although preliminary findings point to a conformational epitope, which agrees with current knowledge of epitopes for most antibodies [88].

The results reported here are based on analysis of many samples from different disease groups and controls, and the results are highly significant. Even so, they should be replicated in larger cohorts and other disease groups, since the presence of the cross-reactive antibodies may correlate with clinical parameters not studied here. Despite of this, power values of 95% for RRMS and 99% for MPN were obtained when compared to HC (Fig. 2), indicating that the results obtained may be representative for larger cohorts as well.

In conclusion, current findings point to that autoantibodies significantly cross-reacting to CALRmut forms and CNX may be common in human sera, and do not react with CALR wt. Collectively, this study indicated that CALR mutations are common and normally do not lead to MPN, presumably due to elimination of cells with mutations. The translational value of this suggests that additional molecular changes may be required for development of MPN, e.g. increased oxidative stress, metabolic changes, escape from immune surveillance, etc. [28]. Also, immune responses to other CALR and CNX interaction partners, e.g. ERp57 should be investigated. In the future, this may contribute to the development of new diagnostic and therapeutic approaches for MPN.

Declaration of Competing Interests

The authors declare no conflict of interests

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Appendix 1. A. Summary of myeloproliferative neoplasm patients including clinical data and antibody reactivity to mutated calreticulin K385 and calnexin. B. Healthy carries of mutated calreticulin identified by population screening [71]. CNX, calnexin; CALRmut, mutated calreticulin; ET, essential thrombocythemia; HCMC, healthy CALR mutation carrier; JAK, Janus kinase; MPL, myeloproliferative leukemia; MPN, myeloproliferative neoplasm; PET, post-essential thrombocythemia; PMF, primary myelofibrosis; PPV, post-polycythemia; PV, polycythemia vera

(Table A, Table B)

Table A

Patient no	Age at diagnosis	Sex	Driver mutation	CALR type	Mutated allele burden	Diagnosis	CALRmut K385 IgG (U/mL)	CNX IgG (U/mL)
MPN 1	72	M	CALR	2		PMF	103.05	175.56
MPN 2	54	M	JAK2			PV	66.64	74.55
MPN 3	48	F	3x neg			ET	15.32	18.55
MPN 4	72	F	CALR	2		PMF (PET-MF)	27.14	192.37
MPN 5	60	M	JAK2			PV	90.97	67.65
MPN 6	58	F	JAK2			ET	157.82	136.40
MPN 7	57	F	3x neg			PV	44.75	46.55
MPN 8	71	M	CALR	1		ET	118.56	133.24
MPN 9	50	F	3x neg			ET	16.54	18.91
MPN 10	51	F	CALR	1		PreMF	25.68	20.90
MPN 11	52	F	CALR	2	44	PreMF	11.63	12.22
MPN 12	56	F	CALR	1	29	PMF	139.18	92.65
MPN 13	41	F	JAK2			PMF	39.96	26.54
MPN 14	48	M	CALR	ND		PMF	75.79	72.27
MPN 15	60	F	CALR	ND		ET	46.74	41.03
MPN 16	72	M	CALR	1	38	PMF	63.40	70.59
MPN 17	48	F	CALR	1		PreMF	9.25	9.84
MPN 18	33	F	CALR	1		PreMF	93.54	79.14
MPN 19	51	M	JAK2			PV	184.61	165.29
MPN 20	39	F	JAK2			PV	64.16	57.59
MPN 21	62	F	JAK2			ET	6.86	10.57
MPN 22	50	F	CALR	ND		PMF (PET-MF)	7.93	7.47
MPN 23	55	F	JAK2			ET	67.02	58.81
MPN 24	38	F	JAK2			PV	15.69	23.19
MPN 25	55	M	JAK2			PMF	16.79	18.65
MPN 26	59	M	JAK2			PV	20.12	18.96
MPN 27	52	M	JAK2			PV	15.08	20.95
MPN 28	56	M	JAK2			PV	17.29	14.50
MPN 29	49	F	CALR	1	13	PMF	116.04	110.28
MPN 30	34	F	CALR	1		PMF	71.41	68.78
MPN 31	54	F	CALR	4	43	PMF	34.45	26.62
MPN 32	59	F	JAK2			ET	16.37	40.50
MPN 33	55	F	MPL			ET	9.20	25.47
MPN 34	44	F	JAK2			PV (PET-PV)	13.21	14.74
MPN 35	71	F	JAK2			PV	63.90	68.62
MPN 36	69	M	JAK2			PMF (PPV-MF)	62.12	80.03
MPN 37	55	M	CALR	1	6	PMF	23.05	44.44
MPN 38	79	F	CALR	1		PMF	179.21	210.67
MPN 39	72	F	CALR	Type-1-like		PMF	78.00	81.36
MPN 40	70	M	CALR	2	38	PreMF	23.79	85.62
MPN 41	52	F	CALR	2		ET	45.79	7.31
MPN 42	57	M	CALR	33, Type-2-like	69	PET-MF	9.20	42.63
MPN 43	58	F	CALR	1		PreMF	8.46	5.11
MPN 44	76	M	CALR	2		PMF	48.55	36.86
MPN 45	65	F	CALR	1		ET	5.68	5.09
MPN 46	54	F	CALR	ND		ET	8.15	13.24
MPN 47	63	F	CALR	1	23	PreMF	94.84	115.25
MPN 48	73	M	CALR	1		PMF	49.60	44.42
MPN 49	72	F	MPL			PMF	6.52	4.35
MPN 50	55	M	JAK2			PV	8.15	50.43

Table B

Carrier no	Age	Sex	Driver mutation	CALR type	Mutated allele burden	CALRmut K385 IgG (U/mL)	CNX IgG (U/mL)
HCMC1	NA	M	CALR	ND	–	20.32	20.41
HCMC2	NA	M	CALR	ND	–	51.35	113.85
HCMC3	NA	F	CALR, JAK2	1	–	14.91	14.28

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