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Elevated levels of a C-terminal agrin fragment identifies a new subset of sarcopenia patients

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ABSTRACT

Sarcopenia is a recently defined medical condition described as age-associated loss of skeletal muscle mass and function. Recently, a transgenic mouse model was described linking dispersal of the neuromuscular junction caused by elevated agrin degradation to the rapid onset of sarcopenia. These mice show a significant elevation of serum levels of a C-terminal agrin fragment (CAF) compared to wild-type littermates. A series of experiments was designed to ascertain the significance of elevated agrin degradation in the development of human sarcopenia. A quantitative Western blot method was devised to detect CAF in sera of humans. A first trial on consenting blood donors (n = 169; age 19–74 years) detected CAF in the limited range of 2.76 ± 0.95 ng/ml. In sarcopenia patients (diagnosed according to clinical and instrumental standards) mean CAF levels were significantly elevated (p = 9.8E10-9; n = 73; age 65–87 years) compared to aged matched controls. Of all sarcopenia patients, 40% had elevated, non-overlapping CAF levels compared to controls. Evidence is presented for a pathogenic role of the agrin/neurotrypsin system in a substantial subset of sarcopenia patients. These patients are characterized by elevated CAF blood levels compared to aged-matched healthy volunteers suggesting the identification of an agrin-dependent form of sarcopenia. Elevated CAF levels in a large subpopulation of sarcopenic patients suggest the existence of a specific form of sarcopenia for which CAF could become a biomarker and a new target for therapeutic interventions. The feasibility of this approach was demonstrated by the development of a small molecule capable of inhibiting neurotrypsin in vitro and in vivo.

1. Introduction

Sarcopenia, often referred to as age-related muscle wasting, is a recently recognized and now exploding geriatric problem (Cruz-Jentoft et al., 2010a), the cost of which in terms of both life expectancy and medical expenses is imposing a heavy burden on the Western Health Care systems (Abellan van Kan, 2009). The impact of sarcopenia on daily life is enormous; hallmarks of sarcopenia are frequent falls, frailty, disability, and shortened life expectancy. Sarcopenia is common in the elderly increasing with age and reaching a 50% incidence peak at the age of 85 (Cruz-Jentoft et al., 2010b). With the increasing life expectancy the incidence of sarcopenia patients is expected to increase substantially in the near future.

There is high need for new biomarkers and effective treatments for sarcopenia (Kimball et al., 2004). Presently, there is a consensus that sarcopenia is a syndrome and may have multiple contributing factors. Nutrition, life style, and hormonal or biochemical changes in muscles particularly the ones affecting energy balance have been suggested to cause sarcopenia (Cannon, 1995; Glass, 2003).

Recently, it has been reported that pre-synaptic expression of the serine protease neurotrypsin in motoneurons produced a sarcopenia-like phenotype in young adult mice (Butikofer et al., 2011). Many post-synaptic and muscular alterations including fragmentation of the neuromuscular junction (NMJ), a reduced number of muscle fibers, increased heterogeneity of fiber size, central nuclei, fiber-type grouping, and an increased proportion of type I fibers were observed. It was suggested that the destabilization of the NMJ through proteolytic cleavage of agrin was the onset of a pathogenic pathway ending in sarcopenia. Agrin, a synaptically located protein (Misgeld et al., 2005; Bezakova et al., 2001; Bezakova and Ruegg, 2003) is a key player for and during initial formation and maintenance of NMJs by regulating the molecular assembly of pre- and post-synaptic structures including aggregates of acetylcholine receptor (AChR) (Bezakova et al., 2001). Cleavage of agrin by neurotrypsin, thereby releasing a soluble 22 kDa C-terminal agrin fragment (CAF) (Stephan et al., 2008) leads to inactivation of agrin and consequently to a dispersal of NMJs (Bolliger et al., 2010). Agrin stands at the beginning of a sophisticated pathway which involves the rearrangement of the

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cytoskeleton beneath the synapse as well as the regulation of the expression of key proteins to facilitate the functioning and maintenance of the NMJ. A main issue of this pathway is the clustering and stabilization of acetylcholine receptors at the NMJ. Binding to LRP-4 agrin triggers the association with MuSK which transfers the signal inside the muscle fiber. MuSK is being self-phosphorylated and the signaling leads via DOK-7 and CRK to diverse activator kinases like Abl, Pak1 and others. Interestingly, the knock out mice of agrin, LRP-4, MuSK, and DOK7 and the double knock out of CRK and CRK-L share the same phenotype with sprouting of nerve terminals in the diaphragm of mice (Lin et al., 2001; Weatherbee et al., 2006; DeChiara et al., 1996; Okada et al., 2006; DeChiara et al., 1996; Hallock et al., 2010). Also, the over-expression of neurotrypsin leads to the same phenotype (Bolliger et al., 2010).

This work for the first time identifies a substantial subgroup of sarcopenia patients suffering from a clear pathogenic mechanism, namely elevated agrin degradation. Agrin-dependent sarcopenia appears to be distinguishable from natural muscle aging and shows a clear neurogenic component. These studies demonstrate that not all sarcopenic patients have the same pathogenic mechanism supporting the concept of multi-factoriality (Cruz-Jentoft et al., 2010b).

2. Materials and methods

2.1. Generation of the human CAF specific monoclonal mouse antibody 28A6H11

Three 6–8 weeks old female Balb/c mice were immunized with 90–150 μg of a C-terminal human agrin fragment in complete Freund's adjuvant with a standard 4 time boost protocol. Cells from the knee lymph knots were prepared and the resulting B-cells were fused with P3-X63-Ag8 mouse myeloma. Supernatants of mother clones were screened by ELISA for positivity. Single clones were generated by a two fold limited dilution.

2.2. Expression and purification of the monoclonal antibody 28A6H11

Hybridoma cells were adapted to serum free ISF-1 medium (BIOCHROME AG) and grown for 5–7 days. Approximately 100 ml conditioned medium was subjected to protein-G sepharose chromatography and eluted with 100 mM citrate buffer pH 2.6.

Positive fractions were pooled and neutralized by an appropriate amount of 2 M Tris. The purified antibody was dialysed against PBS and biotinylated using a NHS biotinylation kit (PIERCE).

2.3. Generation and keeping of SARCO mice

All experiments were performed on C57/Bl6 mice in accordance to Swiss guidelines for proper conduct of animal experiments under supervision of the local authorities. The transgenic human neurotrypsin expressing mice (B6.C3-Tg(PRSS12) 491 Zbz), herein called SARCO mice, are previously described by Stephan et al. (2008) and Butikofer et al. (2011). Genotyping was done as described by Stephan et al. (2008).

2.4. Treatment schedule

5 adult mice were treated with 25 mg/kg NT-1474 in 20% DMSO with PBS (10 ml/kg). As vehicle control another 5 mice were treated with 10 ml/kg of 20% DMSO in PBS. Each mouse received three intraperitoneous injections with 8 hours interval between two injections. 5 h after the last injection the mice were euthanatized with CO_2 and CAF levels of the mice were determined with Western blot.

2.5. Recombinant Agrin fragments and in vitro digestion of human agrin

CAFz0 and CAFz8 were produced and purified as described (Matsumoto-Miyai et al., 2009) using constructs which allowed to cleave off the n-terminal his tag using the prescission cleavage site directly after the tag. After purification via IMAC, the tag was removed by prescission cleavage. Full length human agrin and neurotrypsin were produced and purified as described (Reif et al., 2008). Full length agrin was incubated in 20 mM MOPS, 150 mM NaCl, 5 mM $CaCl_2$, 0.1% PEG, pH 7.5 with neurotrypsin at 37 °C for 2 h and, for a complete digestion for 4 h. As negative control, agrin was incubated without adding neurotrypsin. After digestion the samples were mixed with Lämmli buffer and separated by SDS-PAGE. The gels were stained with Sypro Ruby (Invitrogen) according to the manufacturer's standard protocol. Fluorescence imaging of protein bands was done with a Stella imaging system (Raytest, Germany).

2.6. IC50 determination for human neurotrypsin

The neurotrypsin assay has been performed as described (Reif et al., 2008). In brief, a compound dilution series in DMSO was made and added to the neurotrypsin assays containing a 44 kDa C-terminal agrin fragment (AgC44y0z0) containing the β-cleavage site for neurotrypsin. The reaction was performed for 105 min at 37 °C to ensure measurement at the initial part of the reaction. The reaction was stopped by adding Lämmli buffer and the setups were separated via SDS-PAGE. Quantification of the product bands was made by sypro ruby staining (Invitrogen) and fluorescence determination of the product bands in a Stella imaging system (Raytest).

2.7. Western blot

Human serum samples were stored frozen at −86 °C in 2-ml screw-cap polypropylene tubes. Before use for Western blotting the samples were tempered for 20 min at 37 °C, mixed by inverting them 7 times and centrifuged for 3 min at 16,000 × *g*.

Human serum was diluted 1:20 with 1 × Lämmli buffer and heated for 5 min to 95 °C. Samples (10 μl) were loaded onto 4–12% NUPAGE gels (Inivitrogen). Separated proteins were transferred to PVDF (Invitrogen) membranes by wet blotting for 60 min at 24 V. CAF containing fragments in human serum were detected using the biotinylated monoclonal anti-CAF antibody 28A6H11 described above. As reporter molecule, streptavidin-poly-HRP conjugate (PIERCE) was used. For detection, Chem Glow West substrate (Alpha Innotech) was applied and the chemiluminescence was recorded with a Stella imaging system (Raytest, Germany). CAF concentration in human serum was determined with a known amount of recombinant CAFz0 used as calibrator.

CAF in blood from mice was detected as described above. Briefly, blood was collected by puncture of the lateral tail vein into serum tubes (BD Microtainer) and 1:2 diluted with PBS. The mixture was filtered using 100 kDa cut-off centrifugal filters (Microcon) for 10 min. The flow through was collected and used for analysis by western blot as described above. Murine CAF was detected using the affinity purified polyclonal antibody R139 (Bolliger et al., 2010) and goat anti-rabbit peroxidase conjugate (Sigma Aldrich).

2.8. CAF elevation in SARCO mice

Recently, a mouse model has been reported that reproduces the phenotypes of sarcopenia in humans (Butikofer et al., 2011). The heterozygous mice (SARCO mice) over-express human neurotrypsin under the thy1-2 promoter leading to elevated agrin degradation and a clearly abnormal neuromuscular phenotype. This prompted us to further investigate the link between elevated neurotrypsin activity and the development of sarcopenia in humans, starting from

assessing the reliability of the 22-kDa C-terminal agrin fragment (CAF) that results from agrin cleavage by neurotrypsin at the beta site, as a marker of neurotrypsin activity and/or agrin degradation. This fragment is known to be crucial for the formation of a NMJ. Elevated neurotrypsin activity should result in increased agrin cleavage fragments in general, thus also in increased CAF levels. Elevated agrin degradation is documented in the brain of the neurotrypsin overexpressing mice. However, we developed a method to detect CAF in blood of mice. Indeed, using the above described Western Blot it was possible to detect CAF in both WT littermates and SARCO mice, the latter demonstrating a CAF elevation of approximately 50% compared to the WT (Suppl. Fig. 1). On the contrary, CAF was not detectable in neurotrypsin-deficient mice a fact that strengthens the role of CAF as a marker of the neurotrypsin/agrin system's activity.

2.9. Neurotrypsin cleavage of agrin

To test if agrin cleavage fragments are detectable also in human blood, we investigated in vitro all the possible agrin cleavage fragments which result from neurotrypsin digestions (Suppl. Fig. 2). Neurotrypsin can cleave agrin at two sites, the α-site and the β-site (Reif et al., 2007). The α-site is situated between the first serine/threonine rich segment and the SEA domain, while the β-site is in front of the LG3 domain. Thus, cleavage of agrin by neurotrypsin results in the generation of several agrin fragments. Full length agrin has a protein core size of roughly 215 kDa but runs spread due to its high glycan content above 250 kDa. Complete digestion of agrin (Suppl. Fig. 3 A) results in the generation of an N-terminal agrin fragment (NAF) with a protein core size of about 115 kDa. Due to the high glycan content, it migrates between 150 and 250 kDa in the SDS-gel. By cleavage at the α and β-sites, a middle agrin fragment (MAF) of approximately 80 kDa – depending on the amino acid insertions in the x- and y sites – is generated. It migrates at about 90 kDa in the SDS-gel. Cleavage at the β-site generates CAF (C-terminal agrin fragment) with a molecular weight of 20–22 kDa, depending on the amino acid insertions at the z-site. CAF without any insertion runs at about 22 kDa in an SDS-gel.

Incomplete digestion of agrin with neurotrypsin (Suppl. Fig. 3 B) reveals fragments which are produced if neurotrypsin cleaves only at the α-site or at the β-site. Besides the NAF, MAF and CAF which result from the complete digestion one can observe in addition a C-terminal fragment migrating at about 110 kDa in the gel. This large C-terminal agrin fragment is called AgC110 herein. A large N-terminal fragment of agrin (AgN200) which results by cleavage only at the β-site with a molecular weight of 197–200 kDa size cannot be resolved by SDS gel electrophoresis because it overlaps with the residual full length protein.

Using the CAF specific monoclonal antibody 28A6H11, CAF and AgC110 can be simply detected in human serum (Suppl. Fig. 3 C). Full length agrin was not detectable in serum using this antibody. In addition, we were unable to detect the agrin fragments MAF or AgN200 with the polyclonal R132 antibody (Reif et al., 2007) in human sera. For further studies we then decided to focus on the quantification of CAF, since this fragment is virtually unable to elicit the acetylcholine clustering activity (Bezakova and Ruegg, 2003).

2.10. Normal values for CAF

To substantiate the initial finding that CAF can be measured in human serum and to define the range of normal levels in ostensibly healthy people a control group of 169 consenting Swiss blood donors (BD) from the Zurich area (86 women and 83 men, age 19 to 74 years) was tested for serum CAF levels by Western blotting (Fig. 1). Each sample was measured in duplicate. Over the whole blood donor population CAF levels are in a relatively narrow range of 2.76 ± 0.95 ng/ml. Using the average value ± 2 SD, the normal CAF range falls between 0.86 and 4.66 ng/ml. Of the 6 outliers, one is below the lower limit and 5 are above the upper limit, 4 of these last are older than 50.

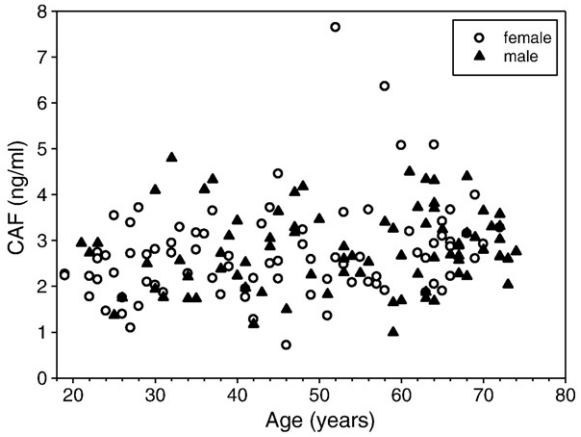

Fig. 1. Scatter diagram of CAF serum levels plotted against age of female (○, n = 86) and male (▲, n = 83) blood donors. Serum CAF levels are in a narrow range in a healthy population.

2.11. Gender and age dependency of CAF

The males' mean (± SD) CAF level is 2.80 ± 0.84 ng/ml compared to the mean of the whole study population (2.76 ± 0.95 ng/ml) and 2.72 ± 1.04 ng/ml for females (Table 1) with no significant gender difference. Due to the age dependency of sarcopenia, we examined whether CAF values vary with age by comparing mean CAF values for each decade represented in the study population (Fig. 2; Table 2). CAF shows the lowest mean value of 2.33 ± 0.69 ng/ml in the youngest group (19–29 y o.) which is also the only group significantly different from the 30–39 y o. ($p = 0.045$), 60–69 y o. ($p = 0.0004$) and 70–74 y o. ($p = 0.0014$) groups. No significant gender difference is observed in each age group (data not shown). In conclusion, there is a significant 15% lower CAF level in young adulthood compared to the mid age groups and slightly higher CAF in people older than 60 (approximately 10% higher than the mid age groups).

2.12. Study populations

A multi-center, non-randomized, open-label, clinical study was designed. After approval from an independent Bioethics Committee at Bangalore, the Institutional Review Boards (IRB) of participating sites and the Indian regulatory authorities, study participants were recruited from geriatric hospitals in Bangalore, Hyderabad and Kalkota, India. Prospective study participants were informed orally and in written form about the purpose of the study and its procedures. After signature of the informed consent, candidates underwent a

Table 1
Characteristics of the analyzed groups. BD: blood donor group; AMC: age matched control group; and SP: sarcopenic patients group.

Group	Gender	n	CAF (ng/ml)	Age (years)
BD	Both	169	2.76 ± 0.95	46.5 ± 15.0
	Male	83	2.80 ± 0.84	49.6 ± 14.6
	Female	86	2.72 ± 1.04	43.4 ± 14.9
AMC	Both	60	2.64 ± 0.97	71.2 ± 5.6
	Male	32	2.90 ± 1.09	73.2 ± 6.7
	Female	28	2.34 ± 0.72	69.0 ± 2.7
SP	Both	73	4.71 ± 2.60	70.8 ± 5.5
	Male	39	5.17 ± 2.79	71.6 ± 5.9
	Female	34	4.18 ± 2.30	69.8 ± 4.9

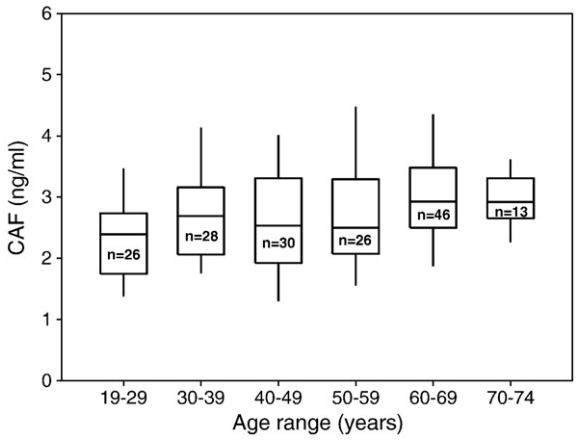

Fig. 2. Box plot representing CAF levels of the BD group categorized by different decades. Boxes are limited by the lower and upper quartiles and the whiskers define the range from 5% to 95% of the data sets. The solid lines in the boxes represent the median value. The numbers of samples of each decade are depicted in the boxes. The CAF level is constant over a wide life span in humans.

preliminary medical screening which included assessment of age (older than 65 years), grip strength, knee strength and a questionnaire regarding performance of daily living and history of falls. Study candidates suffering from co-morbidities such as diabetes, COPD, renal difficulties, osteoporosis and other medical ailments were excluded from the study. For the assessment of grip strength and knee strength the corresponding baseline values published by Park et al. (2007) were used. Assessment of eligibility was done by previously trained local physicians at each hospital.

Out of 750 screened subjects, 350 were eligible for a secondary screening which included dual-energy X-ray absorptiometry (DEXA) of the appendicular skeletal muscle mass (ASMM). On the basis of the published baseline value (Visser et al., 2003) the candidates were divided into the age matched control (AMC) or the sarcopenia patient (SP) group. This latter was formed by subjects with an ASMM smaller than 20%. It was ensured that both sexes were equally represented in the two groups. Study participants older than 65 years were separated into the final aged matched control (AMC, aged from 65 to 88 years) and sarcopenia patient (SP) groups according to accepted standardized diagnostic tools (Cruz-Jentoft et al., 2010a). Inclusion criteria for the SP group were DEXA scan values below −1 (SD). People with DEXA values between −1 and 0 were distributed to the SP or AMC groups based on their grip strength, knee strength and reported difficulties in daily living as frequent falls, difficulties in walking and lifting objects. People with DEXA values >0 were assigned to the AMC group. The final SP group comprised 73 subjects (34 women and 39 men, ages 65 to 87), and the AMC group comprised 60 subjects (28 women and 32 men, ages 65 to 88 years). The study started at the end of May, 2008, and terminated at the beginning of March, 2010. A study flow chart is presented in the supplementary online material (Suppl. Fig. 4).

As reference for a healthy population, in a separate study, blood was obtained from healthy Swiss blood donors after written consent. This independent control group (BD) included 169 subjects (86 women and 83 men, ages 19 to 74 years).

Table 2
BD group divided into decades.

Age group	Gender	n	CAF (ng/ml)
19–29 y	Both	26	2.33 ± 0.69
	Male	6	2.37 ± 0.66
	Female	20	2.23 ± 0.71
30–39 y	Both	28	2.76 ± 0.84
	Male	13	2.88 ± 1.10
	Female	15	2.65 ± 0.55
40–49 y	Both	30	2.61 ± 0.93
	Male	15	2.74 ± 0.91
	Female	15	2.48 ± 0.96
50–59 y	Both	26	2.80 ± 1.42
	Male	12	2.48 ± 0.74
	Female	14	3.06 ± 1.80
60–69 y	Both	46	3.02 ± 0.86
	Male	26	2.97 ± 0.85
	Female	20	3.09 ± 0.88
70–74 y	Both	13	2.97 ± 0.45
	Male	11	2.94 ± 0.48
	Female	2	3.10 ± 0.25

2.13. Anthropometric data acquisition

The study participants' total body mass was determined by scale and their body height was measured by a standard stadiometer. Body mass index (BMI) was calculated as total body mass in kg divided by the square of body height expressed in meters. According to their BMI women were categorized as underweight (<19.1 kg/m^2), normal (19.1–25.8 kg/m^2), or overweight (>25.8 kg/m^2) and men as underweight (<20.7 kg/m^2), normal (20.7–26.4 kg/m^2) or overweight (>26.4 kg/m^2). Threshold data were taken from the Second National Health and Nutrition Examination Survey, (NHANES II) 1976–80.

2.14. Grip and knee strength

Grip and knee strengths were used as indicators of muscle strength (Visser et al., 2003). Grip strength was measured with an isometric Jaymar dynamometer from JLW Instruments, Chicago. Knee strength, i.e. maximal isokinetic strength of knee extensors, was assessed by a Kin-Com 125 AP dynamometer (Chattanooga, TN).

2.15. Statistical analysis

Data were statistically analyzed with the Student's t-test and using the free statistic tool R (version 2.9.0). The data set used contained the variables age, sex, group (BD, SP or AMC), DEXA, height, weight, BMI, grip strength, knee strength and CAF value.

Characteristics of participant groups or subsets thereof are shown as box plots delimited by the lower and upper quartile with whiskers representing the range from 5% to 95% of the data sets. Numbers of participants are depicted in the boxes. Significant differences of the mean values (Student's t-test; $p<0.05$) are marked by asterisks. Values in tables and text are mean values with accompanying standard deviation. Scatter plots between CAF concentration and age are shown for the BD group for both sexes and for the AMC and SP groups.

3. Results

3.1. Design of sarcopenia study

To evaluate the relevance of an enhanced neurotrypsin activity in the development of sarcopenia in humans a clinical study was designed. In total, 73 sarcopenia patients (SP) were identified by the screening method. Another group of 60 people without the indication of sarcopenia served as age matched controls (AMC).

It was made sure that both groups, AMC and SP did not differ in BMI. Also, in the used set of individuals there were no gender specific differences in BMI. For the AMC group, the average BMI was 22.3 ± 2.4 kg/m^2 and for the SP group 22.0 ± 3.8 kg/m^2 (Suppl. Fig. 5).

DEXA scan results were one of the most stringent selection criteria for the grouping of individuals into the AMC or SP group. Thus, the SP group had significantly lower DEXA t-scores (−1.8 ± 1.1) than the

AMC group (0.2 ± 0.9). There was no significant gender specific difference within both groups.

Grip strength measurement revealed significantly weaker grip strength of the females in the SP group compared to the AMC group. The males in the AMC group showed a large heterogeneity in grip strength (24.1 ± 6.8 kg). Therefore, no significant difference to males of the SP group (20.7 ± 3.9 kg) was observed, although the mean values are clearly lower in the SP group.

In addition, knee strength was found to be significantly different between the AMC group (525.6 ± 102.7 N) and the SP group (412.8 ± 134.4 N). There was no gender specific effect in the AMC group while a significant difference was observed in the SP group between males and females ($p = 0.0013$).

3.2. CAF levels in the sarcopenia study

CAF levels of both SP and AMC groups were determined with Western blot. The CAF values from the SP respectively AMC group ranged from 1.51 to 16.68 ng/ml resp. 0.94–6.10 ng/ml. The average CAF level of the SP group was 4.71 ± 2.60 ng/ml. The average of the AMC group was 2.64 ± 0.97 ng/ml (Table 1; Fig. 3). This means that the average CAF level of the SP group is highly significantly elevated compared to the age matched control group AMC ($p \ll 0.00001$). In total, 38% of the SP group (28 out of 73) was above the upper threshold of 4.66 ng/ml as determined by measurement of the blood donor group, whereas in the AMC group, only 4 out of 60 (7%) were above this cut-off level. There is no obvious tendency among the AMC group for an increase of CAF levels with age. In the SP group however, there is a slight tendency for age-associated elevated CAF levels. In this group, the standard deviation was larger than in both other control groups (Fig. 4). The average CAF value of the AMC group is indistinguishable from the blood donor group.

CAF values only slightly differ for the genders in all study groups (Suppl. Fig. 6). For the blood donor group the average CAF values were 2.72 ± 1.04 ng/ml for females and 2.80 ± 0.84 ng/ml for males (not significant). The values for the age matched control group were slightly lower with 2.34 ± 0.72 ng/ml for females and 2.90 ± 1.09 ng/ml for males. In this case one can observe a significant difference ($p = 0.03$). The mean CAF levels for both genders of the sarcopenia patients group were significantly higher with 4.18 ± 2.30 ng/ml for females and 5.17 ± 2.79 ng/ml for males. In this group, the standard deviation was larger than in both other control groups with no significant gender difference.

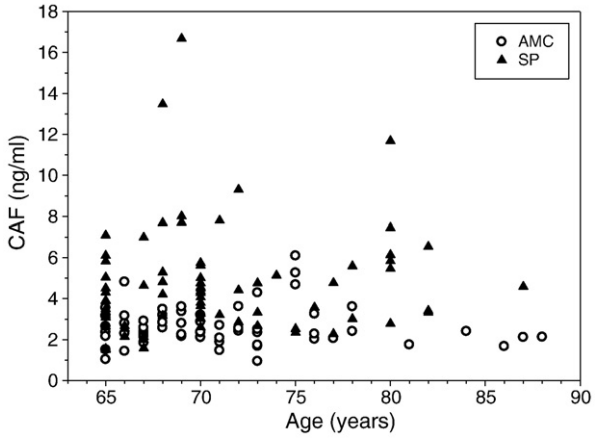

Fig. 3. Scatter plot of CAF serum levels of age matched controls (AMC, ○, n = 60) and sarcopenia patients (SP, ▲, n = 73). Serum CAF levels of sarcopenia patients are elevated compared to an age matched control group.

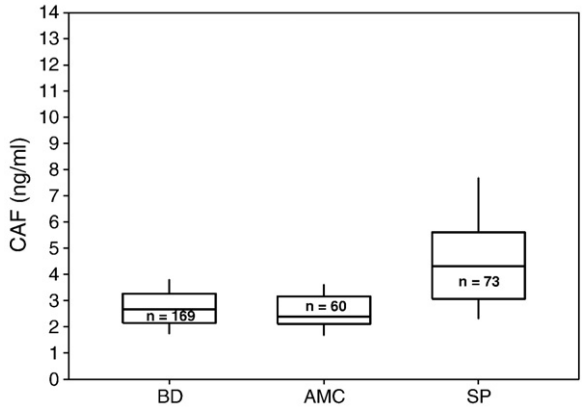

Fig. 4. The CAF level in sarcopenia patients is significantly elevated compared to the BD and AMC groups. Box plot representing CAF levels of the BD, AMC and SP groups. Boxes give the lower and upper quartiles and the whiskers define the range from 5% to 95% of the data sets. The solid lines in the boxes represent the median value. The numbers of samples of each group are depicted in the boxes. BD: blood donor group; AMC: age matched control group; and SP: sarcopenic patients group.

3.3. Development of a small molecule inhibitor

To assess the potential therapeutic intervention we focussed on the protection of agrin from proteoloytic degradation by neurotrypsin. For this we aimed to inhibit neurotrypsin thus protecting agrin's NMJ stabilizing function.

Neurotrypsin is a serine protease but a broad set of known serine protease inhibitors like benzamidine, PMSF, AEBSF, TLCK, aprotinin and leupeptin did not show inhibition of neurotrypsin. It was concluded that neurotrypsin is a very specific protease with a unique substrate binding pocket (Reif et al., 2007). However, a set of lead compounds was identified after screening of a focussed compound library. The neurotrypsin inhibition of these initial compounds was further improved by chemical modifications using a homology model of neurotrypsin. The final compound NT-1474 was shown to be the best neurotrypsin inhibitor reported so far with an IC50 of 570 nM for human neurotrypsin. Treatment of mice with this compound led to a reduction of CAF in murine serum and showed its potency in vivo. After a 3 step dosing with 25 mg/kg NT-1474 within one day the CAF level in serum was reduced by 44% compared to vehicle treated littermates (Fig. 7).

4. Discussion

We have focused our work on the identification and quantification of CAF. This agrin fragment consists basically of the LG3 domain of agrin, which is known to play an essential role in NMJ formation (Bezakova and Ruegg, 2003). CAF itself is not active but absolutely required for NMJ formation. This work clearly identifies CAF as a proteolytic breakdown product of agrin by neurotrypsin. Agrin cleavage at the β-site, inactivates NMJ formation. Currently, neurotrypsin is the sole protease known to generate the agrin fragments CAF and AgC110, as neurotrypsin knock out mice do not contain CAF or AgC110 in blood or tissues (Stephan et al., 2008). Moreover, an increased activity of neurotrypsin in motor neurons led to an increase in CAF blood levels, notably the AgC110 level was not elevated in blood of these mice (unpublished results). In human sera, the same agrin fragments, CAF and AgC110 are detected. They are identical to the ones in mice and are likely to originate from neurotrypsin as well. Finding CAF absence in humans lacking neurotrypsin would bring additional evidence that neurotrypsin is the only source of CAF. Unfortunately, a family with a frameshift mutation in the neurotrypsin gene, leading to an effective neurotrypsin knock out (Molinari et al., 2002) could not be tested for CAF levels.

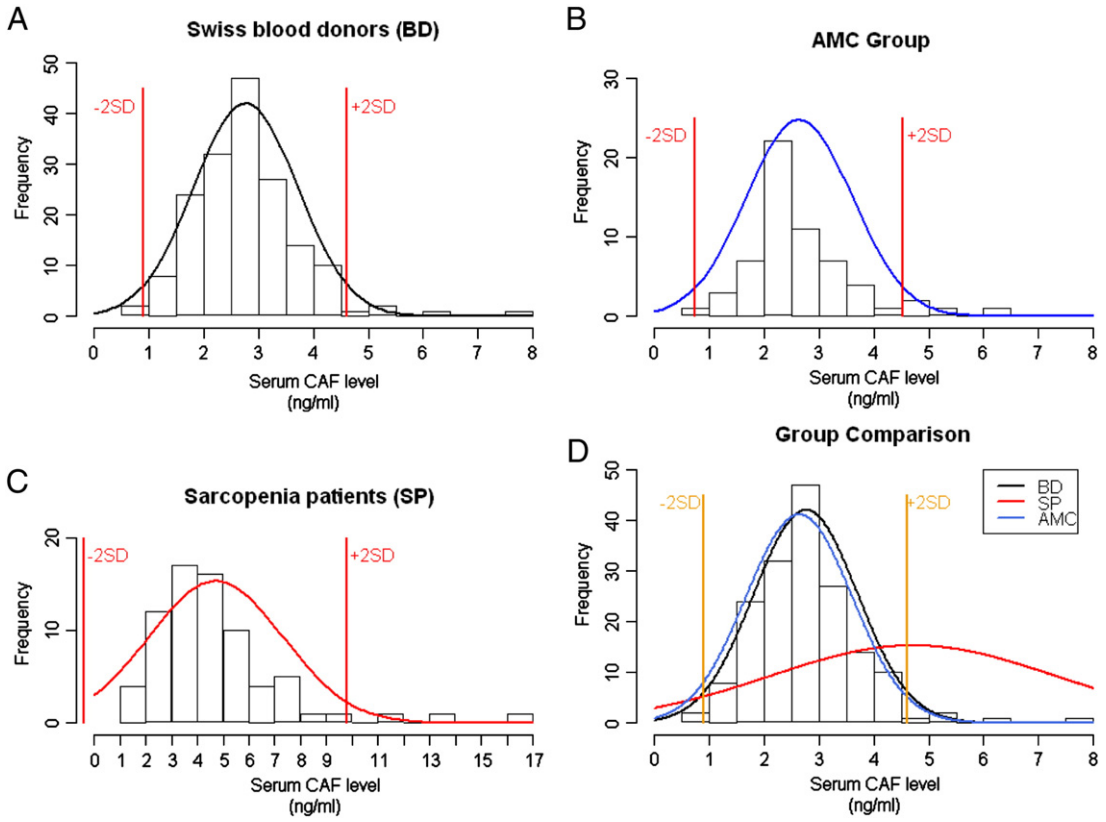

Fig. 5. The CAF levels in the blood donor and age matched control groups lie in a narrow range. Distribution diagrams of CAF levels in 0.5 ng/ml steps of the different study groups. A: BD, B: AMC, C: SP and D: all groups. BD: blood donor group; AMC: age matched control group; and SP: sarcopenic patients group.

The level of CAF detected in serum from randomly selected blood donors lies in a narrow range from 0.86 to 4.66 ng/ml and 96.5% of all measured CAF values are found in this range. Thus, the production of CAF seems to be tightly regulated.

Although no longitudinal CAF studies exist, the detected CAF levels in the different age groups of blood donors suggest a slight increase of CAF over life time. Interestingly, the lowest mean CAF levels are detected in young adult people and the highest mean values are measured in the population older than 60 years.

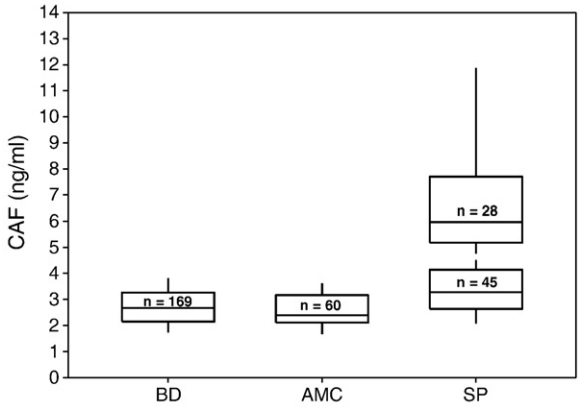

Fig. 6. Box plot representing CAF levels of the BD, AMC and SP groups. The SP group was splitted into a normal level group and a high level group containing all values above the upper threshold of 4.66 ng/ml. Boxes are delimited by the lower and upper quartiles and the whiskers define the range from 5% to 95% of the data sets. The solid lines in the boxes represent the median value. The numbers of samples of each group are depicted in the boxes. BD: blood donor group; AMC: age matched control group; and SP: sarcopenic patients group.

Using common parameters like DEXA scan, grip strength and knee strength for the separation of sarcopenia patients from the control group seem to be an overall good strategy. Nevertheless, it is difficult to get clearly separated groups in respect to all parameters at once. People with low DEXA values can have a high grip or knee strength and vice versa. Thus, the current criteria are not very stringent yet. Interestingly, there seems to be a large variation of strength parameters especially in the male population.

CAF levels in all groups follow a Gaussian distribution (Fig. 5). The BD group shows a mean CAF value of 2.76 ± 0.95 ng/ml (from 0.86 to 4.66 ng/ml i.e. the ± 2SD range). The AMC group is almost identical to the BD group (CAF 2.64 ± 0.97 ng/ml). No value is below the lower threshold of 0.86 ng/ml and 4 values are above the upper threshold of 4.66 ng/ml.

The SP group is clearly different from the BD and AMC groups. The mean CAF value is significantly elevated and the standard deviation is much larger (4.71 ± 2.60 ng/ml). No value is below the lower threshold and 28 out of 73 (38%) are above the upper threshold. The mean CAF level of all values that lie within the normal range is 3.24 ± 0.88 ng/ml. This is almost identical to the corresponding age group in the BD study. Taking into account only values which are above the upper threshold of 4.66 ng/ml the mean CAF value is 7.01 ± 2.80 ng/ml (Fig. 6).

This group of sarcopenia patients are potentially affected by an over-activity of neurotrypsin which leads to NMJ destruction with subsequent loss of muscle mass and function. This group may suffer from agrin dependent sarcopenia while the others may suffer from sarcopenia caused by other reasons like malnutrition, hormonal imbalance, inactivity or other pathophysiological backgrounds.

The mean CAF values of the AMC group are slightly below the values for the blood donor group, although statistically not significant. Individuals from this group were older than 65 years and there was no trend to higher CAF values with increasing age. The reason

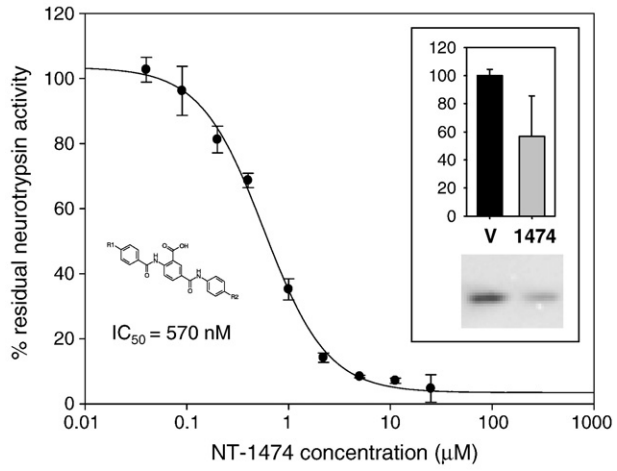

Fig. 7. Structure, IC50 determination and in vivo administration of NT-1474. The structure of the compound is given in the graph, R1 is a heterocycle and R2 is a halogen. The IC50 for neurotrypsin was determined to be 570 nM. Insert the compound was administered intraperitoneal in WT mice and CAF levels were determined by Western blotting. The compound reduces the CAF concentration in blood by more than 40%. V: vehicle control; and 1474: NT-1474 treated mice.

for the lower CAF value can be that the samples from the blood donor group are arbitrarily chosen from the population and one can expect pre-frail people among the selection or people with early developmental stages of sarcopenia although they were regarded as healthy for the donation of blood. In contrast, people from the AMC group were especially selected not to be sarcopenic, which will exclude people suffering from frailty or other muscular diseases. In view of a possible connection of the degree of frailty or sarcopenia with increasing CAF levels, the lower levels in the AMC group clearly make sense.

The significant difference in CAF levels between males and females in the AMC group is somewhat surprising but may reflect different hormonal or physical states in elderly men and women. However, no gender specific difference in the CAF level of the blood donor group was observed.

As mentioned above, there is a high need for biomarkers in sarcopenia (Kimball et al., 2004). This work for the first time identifies a substantial subgroup of sarcopenia patients suffering from a clear pathogenic mechanism, namely elevated agrin degradation. Agrin is a key protein in the development and homeostasis of the neuromuscular junction (Bezakova and Ruegg, 2003). Agrin-dependent sarcopenia appears to be distinguishable from natural muscle aging and shows a clear neurogenic component. These studies demonstrate that not all sarcopenia patients have the same pathogenic mechanism supporting the concept of multi-factoriality (Cruz-Jentoft et al., 2010b).

Our findings open new avenues for specific treatment approaches and have a substantial impact on clinical trial design, treatment of sarcopenia, and monitoring treatment effects. Currently, many researches and geriatricians consider sarcopenia as a sole muscle weakness problem (Borst, 2004). In fact, the true causative problem could be loss of synapses and in this respect sarcopenia bears some similarity to Alzheimer's Disease (Burns et al., 2010).

A promising treatment strategy with the new compound NT-1474 was developed. This compound is able to specifically inhibit neurotrypsin. As CAF levels are a direct measure for neurotrypsin activity, it could be shown that this compound is active in vivo in mice. Future work aims at demonstrating that inhibition of neurotrypsin will reduce the NMJ destabilizing forces in the neuro-muscular system, thus leading to better muscle maintenance and function.

Supplementary data to this article can be found online at doi:10.1016/j.exger.2012.03.002.

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