Inflammation Research

Inflammatory protein response in CDKL5-Rett syndrome: evidence of a subclinical smouldering inflammation

--Manuscript Draft--

Manuscript Number:	INRE-D-16-00277R1
Full Title:	Inflammatory protein response in CDKL5-Rett syndrome: evidence of a subclinical smouldering inflammation
Article Type:	Original Article
Corresponding Author:	Alessio Cortelazzo, PhD University Hospital Azienda Ospedaliera Universitaria Senese Siena, ITALY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University Hospital Azienda Ospedaliera Universitaria Senese
Corresponding Author's Secondary Institution:	
First Author:	Alessio Cortelazzo, PhD
First Author Secondary Information:	
Order of Authors:	Alessio Cortelazzo, PhD
	Claudio De Felice
	Silvia Leoncini, PhD
	Cinzia Signorini, Prof.
	Roberto Guerranti, PhD
	Roberto Leoncini, Prof.
	Alessandro Armini
	Luca Bini, Prof.
	Lucia Ciccoli, Prof.
	Joussef Hayek
Order of Authors Secondary Information:	
Funding Information:	Regione Toscana (Bando Salute 2009; “Antioxidants (ω-3 polyunsaturated Fatty Acids, lipoic acid) supplementation in Rett syndrome: A novel approach to therapy”) (Grant RT no. 142) — MD Joussef Hayek
Abstract:	Background Mutations in the cyclin-dependent kinase-like 5 gene cause a clinical variant of Rett syndrome (CDKL5-RTT). A role for the acute phase response (APR) is emerging in typical RTT caused by methyl-CpG binding protein 2 gene mutations (MECP2-RTT). No information is to date available on the inflammatory protein response in CDKL5-RTT. We evaluated, for the first time, the APR protein response in CDKL5-RTT. Methods Protein patterns in albumin- and IgG-depleted plasma proteome from CDKL5-RTT patients were evaluated by two-dimensional gel electrophoresis/mass spectrometry. The resulting data were related to circulating cytokines and compared to healthy controls or MECP2-RTT patients. The effects of omega-3 polyunsaturated fatty acids (ω-3 PUFAs) were evaluated. Results CDKL5-RTT mutations resulted in a subclinical attenuated inflammation, specifically characterized by an overexpression of the complement component C3 and CD5 antigen-like, both strictly related to the inflammatory response. Cytokine dysregulation featuring a bulk increase of anti-inflammatory cytokines, predominantly

	IL-10, could explain the unchanged erythrocyte sedimentation rate and atypical features of inflammation in CDKL5-RTT. Omega-3 PUFAs were able to counterbalance the pro-inflammatory status. Conclusion For the first time, we revealed the subclinical smouldering inflammation pattern in CDKL5-RTT consisting in the coexistence of an atypical APR coupled with a dysregulated cytokine response.
Response to Reviewers:	POINT-BY-POINT RESPONSE COMMENTS FOR AUTHOR: This is an interesting study which provided details about immune profile in CDKL5-RTT and MECP2-RTT patients. Data demonstrated characteristics of plasma protein, cytokine levels in both variants. Although whether and how these inflammation related proteins/cytokines contribute to the disease was not fully studied, the authors revealed their regulation following omega3 polyunsaturated fatty acids (ω3 PUFAs) supplementation. I would suggest including the data of clinical examination before and during ω3 PUFAs. Such data is very useful and will strengthen the study by indicating whether there is a correlation between the level of inflammatory mediators and clinical symptoms (maybe some improvements in some aspects). If the data is not available, at least, a brief discussion on the significance of the study and future direction will be appreciated. RESPONSE: We thank the reviewer for favourable comments. We do agree to include two clinical illness scoring scales, before and after ω-3 PUFAs treatment, in order to better clarify the clinical improvement following ω-3 PUFAs supplementation, and their possible relationships with cytokines/proteins levels. In particular, we added the data of from RCSS [1] and MBAS [2], due to well known illness severity scores scales from Rett syndrome. We included an extra figure (Figure 2, as composed by 5 separate panels in order to be combined as more convenient for your journal; see attached files). We also implemented the Material and Methods section by adding the methodology for measuring illness severity in RTT. Moreover, we added a short extra paragraph to the Results section. The corresponding data were briefly discussed (see Discussion section). In particular, we evaluated the correlations between the level of the inflammatory mediators and clinical severity (Supplementary Table 3 and 4). References 1. Neul JL, Fang P, Barrish J, Lane J, Caeg EB, Smith EO, et al. Specific mutations in methyl-CpG-binding protein 2 confer different severity in Rett syndrome. Neurology. 2008;70(16):1313-21. 2. FitzGerald PM, Jankovic J, Percy AK. Rett syndrome and associated movement disorders. Mov Disord. 1990;3(5):195-202.

Siena, Nov 6, 2016

Prof. Ji Zhang

Editor, Inflammation Research

RE: Cortelazzo et al. INRE-D-16-00277 – Revision 1

Dear Editor,

Many thanks to the independent reviewer for insightful review of our Ms titled "**Inflammatory protein response in *CDKL5*-Rett syndrome: evidence of a subclinical smouldering",** by Cortelazzo et al.

We do agree to include two clinical illness scoring scales, before and after ω-3 PUFAs treatment, in order to better clarify the clinical improvement following PUFAs supplementation, and their possible relationships with cytokines/proteins levels. In particular, we added the data of from RCSS [1] and MBAS [2], due to well known illness severity scores scales from Rett syndrome. Results were added to the proper section of the manuscript and their implications briefly discussed, along with possible future directions.

Please find enclosed: *i*) a brief point-by-point response and *ii*) the reviewed manuscript with an extra Figure (Figure 2) and two supplementary extra tables (Supplementary Table 3 and 4). Please note that changes to the manuscript were highlighted in red.

We look forward to hearing from you in due course

Best regards

Alessio Cortelazzo

References

1. Neul JL, et al. Neurology. 2008;70(16):1313-21.

2. FitzGerald PM, et al. Mov Disord. 1990;3(5):195-202.

POINT-BY-POINT RESPONSE

COMMENTS FOR AUTHOR:

This is an interesting study which provided details about immune profile in CDKL5-RTT and MECP2-RTT patients. Data demonstrated characteristics of plasma protein, cytokine levels in both variants. Although whether and how these inflammation related proteins/cytokines contribute to the disease was not fully studied, the authors revealed their regulation following omega3 polyunsaturated fatty acids (ω3 PUFAs) supplementation. I would suggest including the data of clinical examination before and during ω3 PUFAs.

Such data is very useful and will strengthen the study by indicating whether there is a correlation between the level of inflammatory mediators and clinical symptoms (maybe some improvements in some aspects). If the data is not available, at least, a brief discussion on the significance of the study and future direction will be appreciated.

RESPONSE:

We thank the reviewer for favourable comments. We do agree to include two clinical illness scoring scales, before and after ω-3 PUFAs treatment, in order to better clarify the clinical improvement following ω-3 PUFAs supplementation, and their possible relationships with cytokines/proteins levels. In particular, we added the data of from RCSS [1] and MBAS [2], due to well known illness severity scores scales from Rett syndrome.

We included an extra figure (Figure 2, as composed by 5 separate panels in order to be combined as more convenient for your journal; see below):

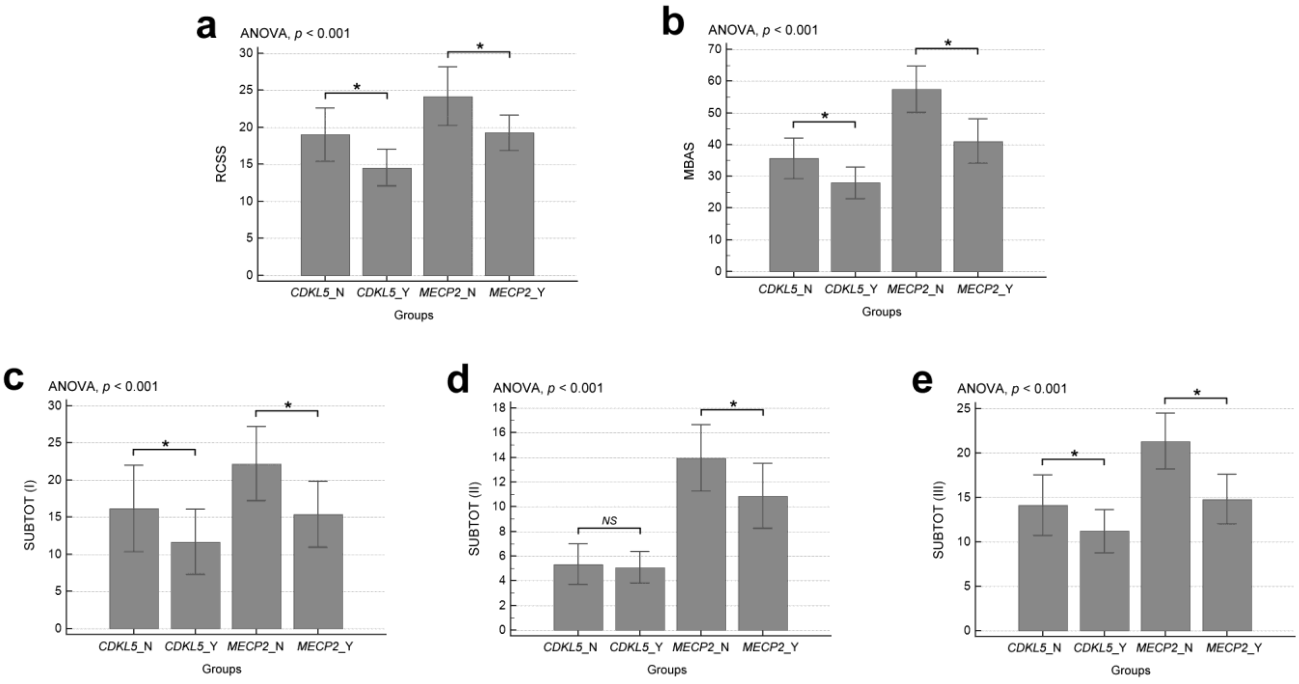

Fig. 2 Clinical illness severity in RTT. The different groups correspond to either *CDKL5*-RTT or *MECP2*-RTT, before ("N" = No) and after ("Y" = Yes) ω-3 PUFAs treatment. Two different scales were evaluated: Rett syndrome Clinical Severity Scale (RCSS) (a) and Motor-Behavioural Assessment Scale (MBAS) (b) with its sub-total scorings ("SUBTOT"), I (c), II (d), and III (e). Two-tailed *p*-value < 0.05 (*); *NS*, not significant.

We also implemented the Material and Methods section by adding the methodology for measuring illness severity in RTT. Moreover, we added a short extra paragraph to the Results section. The corresponding data were briefly discussed (see Discussion section).

In particular, we evaluated the correlations between the level of the inflammatory mediators and clinical severity (Supplementary Table 3 and 4).

References

1. Neul JL, Fang P, Barrish J, Lane J, Caeg EB, Smith EO, et al. Specific mutations in methyl-CpG-binding protein 2 confer different severity in Rett syndrome. Neurology. 2008;70(16):1313-21.

2. FitzGerald PM, Jankovic J, Percy AK. Rett syndrome and associated movement disorders. Mov Disord. 1990;3(5):195-202.

Inflammatory protein response in *CDKL5*-Rett syndrome: evidence of a subclinical smouldering inflammation

Alessio Cortelazzo,[1,2,3,*] Claudio De Felice,[4,*] Silvia Leoncini,[1,5,*] Cinzia Signorini,[5] Roberto Guerranti,[2,3] Roberto Leoncini,[2,3] Alessandro Armini,[6] Luca Bini,[6] Lucia Ciccoli,[5] and Joussef Hayek[1]

[1] Child Neuropsychiatry Unit, University Hospital Azienda Ospedaliera Universitaria Senese (AOUS), Viale M. Bracci 16, 53100 Siena, Italy

[2] Department of Medical Biotechnologies, University of Siena, Via A. Moro 2, 53100 Siena, Italy

[3] Clinical Pathology Laboratory Unit, University Hospital AOUS, Viale M. Bracci 16, 53100 Siena, Italy

[4] Neonatal Intensive Care Unit, University Hospital AOUS, Viale M. Bracci 16, 53100 Siena, Italy

[5] Department of Molecular and Developmental Medicine, University of Siena, Via A. Moro 6, 53100 Siena, Italy

[6] Department of Life Sciences, University of Siena, Via A. Moro 2, 53100 Siena, Italy

[*] These authors contributed equally to this work.

To whom correspondence should be addressed at Child Neuropsychiatry Unit, University Hospital, Azienda Ospedaliera Universitaria Senese (AOUS), Viale M. Bracci 16, 53100 Siena, Italy.
Tel.: +39 0577 234284; Fax: +39 0577 233117; E-mail: alessio.cortelazzo@biologo.onb.it

Abstract

Background Mutations in the *cyclin-dependent kinase-like 5* gene cause a clinical variant of Rett syndrome (*CDKL5*-RTT). A role for the acute phase response (APR) is emerging in typical RTT caused by *methyl-CpG binding protein 2* gene mutations (*MECP2*-RTT). No information is to date available on the inflammatory protein response in *CDKL5*-RTT. We evaluated, for the first time, the APR protein response in *CDKL5*-RTT.

Methods Protein patterns in albumin- and IgG-depleted plasma proteome from *CDKL5*-RTT patients were evaluated by two-dimensional gel electrophoresis/mass spectrometry. The resulting data were related to circulating cytokines and compared to healthy controls or *MECP2*-RTT patients. The effects of omega-3 polyunsaturated fatty acids (ω-3 PUFAs) were evaluated.

Results *CDKL5*-RTT mutations resulted in a subclinical attenuated inflammation, specifically characterized by an overexpression of the complement component C3 and CD5 antigen-like, both strictly related to the inflammatory response. Cytokine dysregulation featuring a bulk increase of anti-inflammatory cytokines, predominantly IL-10, could explain the unchanged erythrocyte sedimentation rate and atypical features of inflammation in *CDKL5*-RTT. Omega-3 PUFAs were able to counterbalance the pro-inflammatory status.

Conclusion For the first time, we revealed a subclinical smouldering inflammation pattern in *CDKL5*-RTT consisting in the coexistence of an atypical APR coupled with a dysregulated cytokine response.

Keywords Rett syndrome; Cyclin-dependent kinase-like 5; Subclinical inflammation; Acute-phase proteins; Polyunsaturated fatty acids; Cytokines

Introduction

Mutations in the X-linked *cyclin-dependent kinase-like 5* (*CDKL5*, OMIM 300203) are known to cause an encephalopathy with a phenotype belonging to the Rett syndromes (RTT, OMIM 312750) [1]. RTT is a severe progressive neurological disorder, usually linked to the *methyl-CpG binding protein 2* (*MECP2*) gene mutations (up to 90-95 % of cases) [2]. Besides the typical *MECP2*-RTT clinical picture [2], several clinical variants have been reported [3]. An early-onset seizures variant (ESV) has been described in 1985 [3], and subsequently related to mutations in *CDKL5* gene [4]. The prevalence and onset of comorbidities in the *CDKL5*-RTT disorder differ significantly from those of *MECP2*-RTT, in terms of epilepsy, scoliosis, gastrointestinal and respiratory problems [5].

An emerging role for inflammation in RTT is reported [6-9]. In particular, prior reports associate *MECP2*-RTT with increased erythrocyte sedimentation rate (ESR), enhanced expression of acute-phase response (APR) proteins and cytokines dysregulation [6, 10].

Apart from an upregulation of Th1- and Th2-related cytokines, as well as increased IL-22 and T-reg cytokine levels, little information on the inflammatory status in *CDKL5*-RTT is available to date [10]. Application of a proteomic approach for identifying possible protein markers holds promise for revealing possible dysregulations of the immune inflammatory homeostasis otherwise not detectable by traditional routine clinical chemistry.

Here, we carried out a detailed expression analysis of the albumin- and IgG-depleted plasma protein patterns in the *CDKL5*-RTT disease in order to highlight changes of less abundant proteins. Protein patterns were related to circulating cytokine levels in *CDKL5*-RTT, and compared with the *MECP2*-RTT picture. In addition, protein changes following omega-3 polyunsaturated fatty acids (ω-3 PUFAs) supplementation were evaluated.

Materials and methods

Subjects

Study participants were 30 RTT female patients with different clinical diagnosis: ESV (n = 15, mean age: 9.4 ± 4.6 years, range: 1.8–17.8) with demonstrated *CDKL5* gene mutation (*CDKL5*-RTT) and typical RTT (*MECP2*-RTT, n = 15, mean age: 9.13 ± 4.3 years, range: 1.6–17.3) with proven *MECP2* gene mutation. RTT diagnosis and inclusion/exclusion criteria were based on the revised RTT nomenclature consensus [11]. Clinical examinations and blood samplings in all the RTT patients were carried out before and after ω-3 PUFAs supplementation, during the routine follow-up study at hospital admission. Clinical illness severity was measured by two different

scoring scales [12, 13]. In particular, the Rett syndrome Clinical Severity Scale (RCSS) is a 13-item scale providing a clinician rating of core symptoms of RTT on a Likert scale of either 0 to 4 or 0 to 5 with a maximum total score of 58 [12]. Regarding the Motor-Behavioural Assessment Scale (MBAS), it is comprised of three sub-scales, i.e., I (behavioural social assessment, 16 items), II (orofacial/respiratory assessment, 7 items) and III (motor/physical signs assessment, 14 items). Each item is related on a scale from 0 to 4, with '0' indicating normal or never, and '4' indicating very severe or constant [13]. Given the specific aims of this study, patients with clinically evident inflammatory conditions, either acute or chronic, were excluded. None of the patients at the time of enrollment were on anti-inflammatory drugs or other known antioxidants. The subjects examined in this study were on a Mediterranean diet. Healthy female control subjects of comparable age (n = 30, mean age: 9.0 ± 4.6 years, range: 1.9–18.9) were also included, and blood samplings were carried out during routine health checks, sports or blood donations. The study was conducted under the approval of the Institutional Review Board of the University Hospital Azienda Ospedaliera Universitaria Senese (AOUS). Informed consents were obtained from either the parents or the legal tutors of the enrolled patients or directly in the case of healthy adults.

Oral supplementation of omega-3 polyunsaturated fatty acids (ω-3 PUFAs)

CDKL5- and *MECP2*-RTT patients were supplemented with ω-3 PUFAs for 12 months, in the form of fish oil before food intake (Norwegian Fish Oil AS, Trondheim, Norway, product number HO320-6; Italian Ministry registration code: 10 43863-Y). ω-3 PUFAs were administered as oil at the dosage of 5 ml daily for patients with b.w. < 20 kg, or 5 ml twice daily with b.w. ≥ 20 kg, corresponding to docosahexaenoic acid (DHA, 22 : 6 ω-3) 36.7 ± 10 mg/kg b.w./day and eicosapentaenoic acid (EPA, 20 : 5 ω-3) 58.5 ± 15.9 mg/kg b.w./day, with a total ω-3 PUFAs of 137.6 ± 37.5 mg/kg b.w./day. Use of ω-3 PUFAs was approved by the AOUS Ethical Committee.

Inflammatory markers

ESR was evaluated as a nonspecific marker of inflammation of widespread clinical use. ESR was measured by an automated system (i.e., "TEST 1") evaluating the aggregation capacity of red blood cells by an infrared ray microphotometer with a light wavelength of 950 nm.

Sample collection and preparation

All samplings from RTT patients and healthy controls were carried out around 8 a.m. after an overnight fasting. Blood was collected in heparinized tubes and all manipulations were carried out

within 2 h after sample collection. Blood samples were centrifuged at 2,400×g for 15 min at 4 °C. The platelet poor plasma was saved and the buffy coat was removed by aspiration. Plasma was used for proteins and cytokines determinations. Plasma samples were stored at -80 °C until assay. For IFN-γ and TGF-β1 evaluations, samples were left to coagulate for 15-30 min at RT and centrifuged at RT for 10 min at 2,000×g. Separated sera were kept in aliquots at -80 °C until the time of assay.

Cytokines enzyme-linked immunosorbent assay

Levels of TNF-α, IL-1β, IFN-γ, IL-12p70, IL-10, IL-5, IL-6, IL-17A, IL-22, IL-8/CXCL8, RANTES/CCL5, I-TAC/CXCL11 and TGF- β1 were quantified in plasma or serum samples from the examined groups by enzyme-linked immunosorbent assay (ELISA), carried out with a commercially available kit (Quantikine, R & D Systems, Minneapolis, MN., USA). The levels of plasma IL-37 were determined using a commercially available ELISA kit (AdipoGen, Switzerland).

Electrophoretic separation of plasma proteins after albumin and IgG depletion

Albumin and IgG were removed using the ProteoPrep Immunoaffinity Albumin & IgG Depletion Kit (Sigma Aldrich), and two-dimensional gel electrophoresis was performed according to Görg [14]. Samples containing 60 µg of protein as determined by Bradford [15], were denatured with 10 ml of a solution containing 10 % of SDS and 2.3 % of dithiothreitol (DTT). Afterwards, samples were combined with 350 ml of solubilizing buffer containing 8 M urea, 2 % of 3-[(3-cholamidopropyl)-dimethylammonium]-1-propane sulfonate, 0.3 % DTT and 2 % of immobilized pH gradient (IPG) buffer, and loaded into 18 cm IPG strips (pH 3-10) nonlinear on an Ettan IPGphor Apparatus system (GE Healthcare), and rehydrated for 7 h. IEF was carried out for a total of 32 kVh. After focusing, the strips were equilibrated with the buffer containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 2 % w/v SDS, 30 % v/v glycerol, and 1 % w/v DTT for 15 min. Subsequently, strips were equilibrated again with the same equilibration buffer described above, except that it contained 4 % w/v iodoacetamide instead of DTT and a trace of bromophenol blue. IPG strips and a molecular weight standard were embedded at the top of a 1.5 mm thick vertical polyacrylamide gradient gel (8-16 % T) using 0.5 % w/v agarose and run at a constant current of 40 mA/gel at 20 °C. The second dimension was performed on an EttanDalt Six Electrophoresis system (GE Healthcare). Each sample was carried out in triplicate under the same conditions.

Protein identification

After mass spectrometry compatible silver staining, the preparative gel was matched to the master gel in the analytical gel match set [16]. A spot-picking list was generated and exported to Ettan Spot Picker (GE Healthcare). The spots were excised and delivered into 96-well microplates where they were destained and dehydrated with acetonitrile (ACN) for subsequent rehydration with trypsin solution. Tryptic digestion was carried out overnight at 37 °C. Each protein spot digest (0.75 ml) was spotted into the MALDI instrument target an allowed to dry. Then 0.75 ml of the instrument matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid in 50 % ACN and 0.5 % v/v trifluoroacetic acid) was applied to dried samples and dried again. Mass spectra were obtained using an ultrafleXtreme MALDI-ToF/ToF (Bruker Corporation, Billerica, MA, USA), as previously described [17]. After tryptic peptide mass acquisition, mass fingerprint searching was carried out in Swiss-Prot/TREMBL and NCBInr databases using MASCOT (Matrix Science, London, UK, http://www.matrixscience.com). A mass tolerance of 100 ppm was allowed and only one missed cleavage site was accepted. Alkylation of cysteine by carbamidomethylation was assumed as a fixed modification, whereas oxidation of methionine was considered a possible modification. Criteria used to accept identifications included the extent of sequence coverage, number of matched peptides and probabilistic scores. Tryptic digests that did not produce MALDI-TOF unambiguous identifications were subjected to ESI-Ion-trap MS/MS peptide sequencing on a nanospray/LCQ DECA Ion Trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA).

Image and data analysis

Gels imaging was performed by using ImageMaster 2D Platinum v7.0 software (GE Healthcare). A reference gel for each group was defined for the comparative analyses. The background was subtracted from all gels using the average on-boundary method. Spot volume was expressed as a ratio of the percentage volume (%V) detected from the entire gel to minimize differences between samples (i.e., normalization). Unmatched spots or spots with significantly different %V were considered as differently expressed. Data were expressed as mean ± SD or medians [95% C.I. for median] as appropriate. Statistical analysis of protein variations was performed using multiple *t*-test with a False Discovery Rate (*q*) of 0.05. Differences between groups were tested using Kruskal-Wallis test, or one-way ANOVA, with Dunn's or Holm-Sidak's multiple comparisons tests for post hoc analyses. A two-tailed *p*-value of less than 0.05 was considered statistically significant. The statistical software Graph-Pad Prism v6.01 (GraphPad Software, Inc., La Jolla, CA, USA), and MedCalc v12.1.4 software package (MedCalc Software, Mariakerke, Belgium) were used.

Results

Inflammatory plasma protein response in *CDKL5*-Rett syndrome (*CDKL5*-RTT)

In *CDKL5*-RTT, proteins with increased expression included: alpha-1-antitrypsin (A1AT), alpha-1B-glycoprotein (A1BG), complement component C3 (CO3), serum transferrin (TRFE), Ig alpha-1-chain C region (IGHA1), Ig mu chain C region (IGHM), CD5 antigen-like (CD5L), inter-alpha-trypsin inhibitor H4 (ITIH4) and fibrinogen alpha chain (FIBA), while clusterin (CLUS) and apolipoprotein A1 (APOA1) were significantly underexpressed as compared to control plasma samples (Figure 1, panels a and b; Table 1; Supplementary Table 1).

In *MECP2*-RTT plasma samples, increased expression was observed for ceruloplasmin (CERU), A1AT, A1BG, TRFE, IGHA1, IGHM, ITIH4 and FIBA, whereas CLUS and APOA1 were significantly decreased as compared to control plasma samples (Figure 1, panel c; Table 1).

In the *CDKL5*-RTT plasma protein pattern, an underexpression was observed for CERU and A1AT (spot #2), while CO3, CD5L, CLUS and APOA1 were significantly increased as compared with *MECP2*-RTT. Overall, CO3 and CD5L appeared to characterize the *CDKL5*-RTT pattern from that of *MECP2*-RTT.

ESR was not significantly changed in *CDKL5*-RTT (mean value ± SD: 12.6 ± 5.6 mm/h) as compared to healthy subjects (9.2 ± 2.5 mm/h, $P = 0.0708$). In contrast, ESR was significantly increased in *MECP2*-RTT (34.6 ± 13 mm/h, $P < 0.0001$). ESR was positively related to CERU, A1AT, A1BG, TRFE, IGHA1, IGHM, ITIH4 and FIBA. In contrast, ESR was inversely related to CLUS and APOA1. No statistically significant relationships were observed between ESR and CO3 or CD5L ($P \geq 0.055$) (Supplementary Table 2).

Circulating cytokine patterns

In *CDKL5*-RTT, cytokines with an increased level include TNF-α, IL-1β, IFN-γ, IL-12p70, IL-10, IL-5 and IL-37, while I-TAC levels were significantly decreased, as compared to the cytokine levels from control samples (Table 2).

In *MECP2*-RTT samples, increased levels were observed for TNF-α, IL-5, IL-6, IL-8 and IL-37, whereas IL-22 and I-TAC levels were significantly decreased, as compared to those of control samples. In the *CDKL5*-RTT, increased levels were observed for IL-1β, IFN-γ, IL-12p70, IL-10, IL-22 and TGF-β1, whereas IL-17A, IL-8 and IL-37 were significantly decreased as compared to the cytokine levels from *MECP2*-RTT samples.

Correlations between plasma proteins and circulating cytokines

In Table 3, the correlation matrix between differentially expressed proteins and circulating cytokines is illustrated. Proteins with a well-defined role in APR (positive or negative) show several significant correlations with circulating cytokine. A slight majority (about 66% i.e., 63/96 significant correlations) of biological consistent relationships (i.e., positive APR proteins *vs.* pro-inflammatory cytokines and negative APR proteins *vs.* anti-inflammatory cytokines) were detected faced to about one third of inconsistent correlations (approximately 34% i.e., 33/96 significant relationship). Notably, a significant positive correlation between IL-10, a well known anti-inflammatory/regulatory cytokine [18], as well as CO3, a characterizing protein for the *CDKL5*-RTT pattern, was detected. Mixed relations were observed between pro-/anti-inflammatory cytokines and CD5L, another distinctive *CDKL5*-RTT protein. Likewise, a mixed behaviour was observed for the CLUS correlation matrix. Finally, IGHA1 and IGHM positively correlated with IL-12p70.

Correlations between inflammatory mediators and clinical illness severity in RTT

Correlations between illness clinical severity and inflammatory mediators are shown in Supplementary Table 3 and 4. Positive relationships with illness severity (RCSS and/or MBAS) were observed for TNF-α, IL-5, IL-6, IL-17A, IL-8 and IL-37. Whereas inverse relationships for IL-22, RANTES, I-TAC and TGF-β1.

Generally, positive correlations were observed between positive APR reactants (i.e., CERU, A1AT A1BG, CO3, ITIH4 and FIBA) and global illness severity (RCSS and/or MBAS). Mixed correlations were evidenced for negative APR reactants (i.e., TRFE and APOA1). A positive correlation between CD5L and RCSS was detectable. A strong inverse relationship with illness severity was evidenced for CLUS, while positive relationships were detected for IGHA1and IGHM. Interestingly, differential correlations were observed beteen inflammatory mediators and MBAS sub-scores (Supplementary Table 3 and 4).

Effects of ω-3 PUFAs on clinical illness severity in *CDKL5*-RTT and *MECP2*-RTT

A marked improvement in illness severity was observed after ω-3 PUFAs supplementation in both *CDKL5*-RTT and *MECP2*-RTT (Figure 2, panels a and b). In particular, from the MBAS sub-scales, ω-3 PUFAs appear to act effectively on all the assessed areas in *MECP2*-RTT, although they seem to act mainly on behavioural, social interaction and motor/physical signs in *CDKL5*-RTT (Figure 2, panels c-e).

Modulatory effects of ω-3 PUFAs on inflammatory proteins in *CDKL5*-RTT

Supplementation with ω-3 PUFAs appears to counterbalance the pro-inflammatory status in *CDKL5*-RTT. Specifically in *CDKL5*-RTT, changes in the expression of 6 proteins (i.e., A1AT spots #2 and #8, TRFE spot #6, IGHM, CD5L, ITIH4 spot #15 and FIBA) were partially rescued, and expression of 4 proteins (i.e., A1BG, CO3, IGHA1 and TRFE spot #11) was fully rescued by ω-3 PUFAs. Moreover, expression of CO3 and CD5L, characterizing the plasma protein pattern of *CDKL5*-RTT, was rescued by the fatty acids supplementation. On the other hand, in *MECP2*-RTT, the expression of 8 proteins (i.e., CERU, A1AT spots # and #8, A1BG, TRFE, IGHM, CLUS, ITIH4 spot #15 and FIBA) was partially rescued, with IGHA1 expression being fully normalized (Figure 1, panels d and e; Table 4).

Among the tested cytokines, only TNF-α was fully normalized in *CDKL5*-RTT following the ω-3 PUFAs treatment. In *MECP2*-RTT, the levels of four cytokines (i.e., TNF-α, IFN-γ, IL-12p70 and I-TAC) were comparable to those of control samples. Likewise, IL-37 was significantly decreased, although not normalized, after ω-3 PUFAs. Paradoxically, IL-6 levels significantly increased after ω-3 PUFAs in *MECP2*-RTT, whereas the increase was not statistically significant in *CDKL5*-RTT (Table 2).

Discussion

Our findings indicate, for the first time, the presence of a subclinical and attenuated APR picture in *CDKL5*-RTT showing evident similarities and differences with *MECP2*-RTT. Shared features are represented by the increased positive APR reactants A1AT, A1ABG, ITIH4 and FIBA, and the negative reactant TRFE, associated with a decrease in APOA1 and CLUS. Apart from the typical APR function, these proteins are also involved in immunity. In particular, A1AT and ITIH4, two well known protease inhibitors involved in tissue protection, apoptosis, cellular senescence and modulation of immunity, [19, 20]. Likewise, A1BG and CLUS shown close connections with the immune response. Specifically, A1BG, a plasma glycoprotein with still unknown functions, is implicated in immune response inflammation processes, and shows sequence similarities to the variable regions of some immunoglobulin supergene family member proteins [21]. CLUS is involved in apoptosis, complement system regulation, inflammatory and immunological processes and autoimmunity [22]. Furthermore, its action in attenuating inflammation suggests a possible role of the relative deficit in CLUS in the atypical inflammatory status generally observed in RTT. Of interest, we evidenced an overexpression of FIBA, a chemoattractant for inflammatory cell infiltration in the immune response acting in the cross-talk between coagulation and inflammation

[23]. In addition, immunoglobulins (IGHA1 and IGHM) were overexpressed in both conditions. Notably, IGHA1 and IGHM levels positively correlate with IL-12p70, a cytokine derived from B-cells and targeting T-cells and NK-cells where it induces Th1 cell differentiation and cytotoxicity [24]. These data confirm a close interaction between inflammatory response and immunity in RTT [10, 25]. APOA1, a negative APR reactant and the principal component of high-density lipoproteins, shows an impressive portfolio of anti-inflammatory mechanisms at the interface of vascular inflammation and inflamed tissue [26]. Its properties list includes inhibition of endothelial cell adhesion molecule expression, myeloid lineage cell proliferation, as well as expression of chemokine receptors and cytokines in synovial lining cells, and neutrophil ingress into inflamed tissues [26]. Here, we observed a paradoxical increase in TRFE in both *CDKL5*- and *MECP2*-RTT plasma protein patterns, which could be related to the previously observed increase in plasma non-protein bound-iron (free redox iron) levels in RTT [10].

A key novel finding for the *CDKL5*-RTT plasma protein pattern is represented by the increase in CO3 and CD5L, which specifically characterize the *CDKL5*-RTT inflammatory response.

As the most abundant component of the complement system, CO3 is indispensable for all three pathways of the complement system activation [27]. Of note, CO3 is an APR proteins member whose levels of expression are known to be regulated by cytokines, such as TNF-α, IL-1β and IL-6 [27]. Remarkably, CO3 is produced by human monocyte-derived macrophages [28].

CD5L, the other characterizing protein in *CDKL5*-RTT, is a macrophage-secreted glycoprotein mainly expressed in lymphoid and inflamed tissues, regulating inflammatory response processes [29]. CD5L is a key regulator of tissue macrophages homeostasis [30]. In particular, CD5L is known to prevent toll-like receptors-induced TNF-α and IL-1β secretion, with a concomitant increase in IL-10 levels, thereby downregulating the tissue macrophages inflammatory reaction [30]. The evidenced positive correlation between CD5L and IL-10 is in line with its known biological function, thus suggesting a general hyperactivity of the macrophage component of *CDKL5*-RTT.

A further distinctive feature for the inflammatory response in *CDKL5*-RTT appears to be the missing increase in CERU, the major copper-carrying protein in the blood of which the plasma level nearly doubles in response to acute and chronic inflammatory processes, trauma or infection, with a ferroxidase activity playing a role in iron metabolism [31]. Interestingly, a strong inverse correlation was found between CERU and IL-10, thus underlying possible mechanism for the lack of increase in this positive APR protein in *CDKL5*-RTT.

IL-10 is the mostly characterizing cytokine in the inflammatory pattern of *CDKL5*-RTT, thus confirming prior data [10]. IL-10, a cytokine with multiple and pleiotropic effects in immune regulation and inflammation, is known to downregulate the expression of Th1 cytokines and the co-stimulatory molecules on macrophages, as well as inhibit NF-κB activity, regulate cyclooxygenase 2 expression and modulate the Janus kinase/signal transducers and activators of transcription (JAK-STAT) signaling pathway [18, 32, 33].

Apart from IL-10, TNF-*α* and I-TAC are the cytokines mostly associated with plasma protein changes. In particular, TNF-*α* shows biologically consistent correlations with seven APR proteins, against the biological apparently inconsistent positive relationship with TRFE. On the other hand, I-TAC shows inconsistent correlations with five APR proteins, against a consistent one (i.e., TRFE). Therefore, our findings reinforce the key concept of a global dysregulation of the inflammatory response in RTT [10]. The observed cytokine dysregulation featuring a bulk increase of anti-inflammatory cytokines, predominantly IL-10, could explain the unchanged ESR values and the atypical features of the inflammatory response in *CDKL5*-RTT.

Our correlation matrix data indicate a likely contribution of inflammatory mediators and/or differentially expressed proteins to clinical phenotype in RTT.

Beneficial effects of ω-3 PUFAs supplementation in *MECP2*-RTT have been previously reported [34-38]. In the present study, protein expression of A1AT (spots #2 and #8), ITH4 (spot #15) and FIBA were partially rescued by ω-3 PUFAs supplementation in both *CDKL5*- and *MECP2*-RTT. However, differentially modulatory effects of ω-3 PUFAs in treated *CDKL5*-RTT are to be emphasized since the fatty acids supplementation leads to a complete rescue for CO3 and partial rescue for CD5L against unchanged expression for these proteins in treated *MECP2*-RTT. The observed improvement in illness severity following ω-3 PUFAs suggests a close association between levels of the inflammatory mediators and specific clinical manifestations of the disease.

Overall, the subclinical smouldering inflammation observed in *CDKL5*-RTT consists in a complex molecular picture, including the coexistence of an atypical APR coupled with a dysregulated cytokine response.

Acknowledgements

Our heartily thanks go to the professional singer Matteo Setti (www.matteosetti.com) and the internationally recognized illustrator Roberto Innocenti (www.robertoinnocenti.com) for continued support and the sensitization work towards Rett syndrome; the Italian importer for the Norwegian Fish Oil, Dr. Ezio Toni (Transforma AS Italia, Forlì, Italy); and Dr. Roberto Faleri (Central Medical Library) for online bibliographic assistance. A special thank goes to Rett girls and their families. This work was partially supported by a grant to J.H. from the Regione Toscana (Bando Salute 2009; "Antioxidants (ω-3 polyunsaturated Fatty Acids, lipoic acid) supplementation in Rett syndrome: A novel approach to therapy," Grant RT no. 142).

Conflict of interest

We declare no conflicts of interest regarding the publication of this paper.

References

1. Weaving LS, Christodoulou J, Williamson SL, Friend KL, McKenzie OL, Archer H, et al. Mutations of *CDKL5* cause a severe neurodevelopmental disorder with infantile spasms and mental retardation. Am J Hum Genet. 2004;75(6):1079-93.

2. Chahrour M, Zoghbi HY. The story of Rett syndrome: from clinic to neurobiology. Neuron. 2007;56(3):422-37.

3. Hanefeld F. The clinical pattern of the Rett syndrome. Brain Dev. 1985;783:320-25.

4. Bahi-Buisson N, Nectoux J, Rosas-Vargas H, Milh M, Boddaert N, Girard B, et al. Key clinical features to identify girls with *CDKL5* mutations. Brain. 2008;131(Pt 10):2647-61.

5. Mangatt M, Wong K, Anderson B, Epstein A, Hodgetts S, Leonard H, et al. Prevalence and onset of comorbidities in the *CDKL5* disorder differ from Rett syndrome. Orphanet J Rare Dis. 2016;11:39. doi:10.1186/s13023-016-0418-y.

6. Cortelazzo A, De Felice C, Guerranti R, Signorini C, Leoncini S, Pecorelli A, et al. Subclinical inflammatory status in Rett syndrome. Mediators Inflamm. 2014;2014:480980. doi:10.1155/2014/480980.

7. Cronk JC, Derecki NC, Ji E, Xu Y, Lampano AE, Smirnov I, et al. Methyl-CpG binding protein 2 regulates microglia and macrophage gene expression in response to inflammatory stimuli. Immunity. 2015;42(2):679-91.

8. Derecki NC, Privman E, Kipnis J. Rett syndrome and other autism spectrum disorders-brain diseases of immune malfunction? Mol Psychiatry. 2010;15(4):355-63.

9. O'Driscoll CM, Lima MP, Kaufmann WE, Bressler JP. Methyl CpG binding protein 2 deficiency enhances expression of inflammatory cytokines by sustaining NF-κB signaling in myeloid derived cells. J Neuroimmunol. 2015:283:23-9.

10. Leoncini S, De Felice C, Signorini C, Zollo G, Cortelazzo A, Durand T, et al. Cytokine dysregulation in *MECP2*- and *CDKL5*-related Rett syndrome: relationships with aberrant redox homeostasis, inflammation, and ω-3 PUFAs. Oxid Med Cell Longev. 2015;2015:421624. doi:10.1155/2015/421624.

11. Neul JL, Kaufmann WE, Glaze DG, Christodoulou J, Clarke AJ, Bahi-Buisson N, et al. Rett syndrome: revised diagnostic criteria and nomenclature. Ann Neurol. 2010;68(6):944-50.

12. Neul JL, Fang P, Barrish J, Lane J, Caeg EB, Smith EO, et al. Specific mutations in methyl-CpG-binding protein 2 confer different severity in Rett syndrome. Neurology. 2008;70(16):1313-21.

13. FitzGerald PM, Jankovic J, Percy AK. Rett syndrome and associated movement disorders. Mov Disord. 1990;3(5):195-202.

14. Görg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, et al. The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis. 2000;21(6):1037-53.

15. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-54.

16. Mortz E, Krogh TN, Vorum H, Görg A. Improved silver staining protocols for high sensitivity protein identification using matrix-assisted laser desorption/ionization-time of flight analysis. Proteomics. 2001;1(11):1359-63.

17. Hellman U, Wernstedt C, Góñez J, Heldin CH. Improvement of an "In-Gel" digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing. Anal Biochem. 1995;224(1):451-55.

18. Taylor A, Verhagen J, Blaser K, Akdis M, Akdis CA. Mechanisms of immune suppression by interleukin-10 and transforming growth factor-beta: the role of T regulatory cells. Immunology. 2006;117(4):433-42.

19. Hunt JM, Tuder R. Alpha 1 anti-trypsin: one protein, many functions. Curr Mol Med. 2012;12(7):827-35.

20. Lee KY, Feng PH, Ho SC, Chuang KJ, Chen TT, Su CL, et al. Inter-alpha-trypsin inhibitor heavy chain 4: a novel biomarker for environmental exposure to particulate air pollution in patients with chronic obstructive pulmonary disease. Int J Chron Obstruct Pulmon Dis. 2015;10:831-41.

21. Cubedo J, Padró T, Badimon L. Coordinated proteomic signature changes in immune response and complement proteins in acute myocardial infarction: the implication of serum amyloid P-component. Int J Cardiol. 2013;168(6):5196-5204.

22. García-Rodríguez S, Arias-Santiago S, Perandrés-López R, Orgaz-Molina J, Castellote L, Buendía-Eisman A, et al. Decreased plasma levels of clusterin in patients with psoriasis. Actas Dermosifiliogr. 2013;104(6):497-503.

23. Jennewein C, Tran N, Paulus P, Ellinghaus P, Eble JA, Zacharowski K. Novel aspects of fibrin(ogen) fragments during inflammation. Mol Med. 2011;17(5-6):568-73.

24. Seruga B, Zhang H, Bernstein LJ, Tannock IF. Cytokines and their relationship to the symptoms and outcome of cancer. Nat Rev Cancer. 2008;8(11):887-99.

25. De Felice C, Leoncini S, Signorini C, Cortelazzo A, Rovero P, Durand T. Rett syndrome: an autoimmune disease? Autoimmun Rev. 2016;15(4):411-16.

26. Terkeltaub R. Apolipoprotein a-I at the interface of vascular inflammation and arthritis. Arterioscler Thromb Vasc Biol. 2014;34(3):474-6.

27. Jeon B, Kim HR, Kim H, Chung DK. In vitro and in vivo downregulation of C3 by lipoteichoic acid isolated from Lactobacillus plantarum K8 suppressed cytokine-mediated complement system activation. FEMS Microbiol Lett. 2016;363(14). doi:10.1093/femsle/fnw140.

28. Vaisar T, Pennathur S, Green PS, Gharib SA, Hoofnagle AN, Cheung MC, et al. Shotgun proteomics implicates protease inhibition and complement activation in the antiinflammatory properties of HDL. J Clin Invest. 2007;117(3):746-56.

29. Sanjurjo L, Amézaga N, Vilaplana C, Cáceres N, Marzo E, Valeri M, et al. The scavenger protein apoptosis inhibitor of macrophages (AIM) potentiates the antimicrobial response against Mycobacterium tuberculosis by enhancing autophagy. PLoS One. 2013;8(11):e79670. doi:10.1371/journal.pone.0079670.

30. Sanjurjo L, Amézaga N, Aran G, Naranjo-Gómez M, Arias L, Armengol C, et al. The human CD5L/AIM-CD36 axis: A novel autophagy inducer in macrophages that modulates inflammatory responses. Autophagy. 2015;11(3):487-502.

31. Samygina VR, Sokolov AV, Bourenkov G, Petoukhov MV, Pulina MO, Zakharova ET, et al. Ceruloplasmin: macromolecular assemblies with iron-containing acute phase proteins. PLoS One. 2013;8(7):e67145. doi:10.1371/journal.pone.0067145.

32. Turner MD, Nedjai B, Hurst T, Pennington DJ. Cytokines and chemokines: at the crossroads of cell signalling and inflammatory disease. Biochim Biophys Acta. 2014;1843(11):2563-82.

33. Akdis M, Burgler S, Crameri R, Eiwegger T, Fujita H, Gomez E, et al. Interleukins, from 1 to 37, and interferon-γ: receptors, functions, and roles in diseases. J Allergy Clin Immunol. 2011;127(4):701-21.

34. De Felice C, Signorini C, Durand T, Ciccoli L, Leoncini S, D'Esposito M, et al. Partial rescue of Rett syndrome by ω-3 polyunsaturated fatty acids (PUFAs) oil. Genes Nutr. 2012;7(3):447-58.

35. Signorini C, De Felice C, Leoncini S, Durand T, Galano JM, Cortelazzo A, et al. Altered erythrocyte membrane fatty acid profile in typical Rett syndrome: effects of omega-3 polyunsaturated fatty acid supplementation. Prostaglandins Leukot Essent Fatty Acids. 2014;91(5):183-93.

36. Ciccoli L, De Felice C, Paccagnini E, Leoncini S, Pecorelli A, Signorini C, et al. Morphological changes and oxidative damage in Rett syndrome erythrocytes. Biochim Biophys Acta. 2012;1820(4):511-20.

37. Cortelazzo A, De Felice C, Guerranti R, Leoncini R, Barducci A, Leoncini S, et al. Erythrocyte cytoskeletal-plasma membrane protein network in Rett syndrome: effects of ω-3 polyunsaturated fatty acids. Curr Proteomics. 2015;12(4):217-26.

38. De Felice C, Cortelazzo A, Signorini C, Guerranti R, Leoncini S, Pecorelli A, et al. Effects of ω-3 polyunsaturated fatty acids on plasma proteome in Rett syndrome. Mediators Inflamm. 2013;2013:723269. doi:10.1155/2013/723269.

Figure captions

Fig. 1 Plasma protein patterns from healthy subjects (**a**), untreated *CDKL5*-RTT patients (**b**), untreated *MECP2*-RTT (**c**) patients, and as a function of mutated gene (*CDKL5* or *MECP2*) and ω-3 PUFAs supplementation in *CDKL5*-RTT (**d**) and *MECP2*-RTT (**e**). Spot numbers that denote identified plasma proteins by MS are listed in Tables 1, 3 and 4, and supplementary Tables 1 and 2. Molecular weight (MW, kDa) and pH markers are indicated.

Fig. 2 Clinical illness severity in RTT. The different groups correspond to either *CDKL5*-RTT or *MECP2*-RTT, before ("N" = No) and after ("Y" = Yes) ω-3 PUFAs treatment. Two different scales were evaluated: Rett syndrome Clinical Severity Scale (RCSS) (**a**) and Motor-Behavioural Assessment Scale (MBAS) (**b**) with its sub-total scorings ("SUBTOT"), I (**c**), II (**d**), and III (**e**). Two-tailed p-value < 0.05 (*); *NS*, not significant.

Figure 1, panel (a)

Click here to download Figure Figure, panel (a).tif

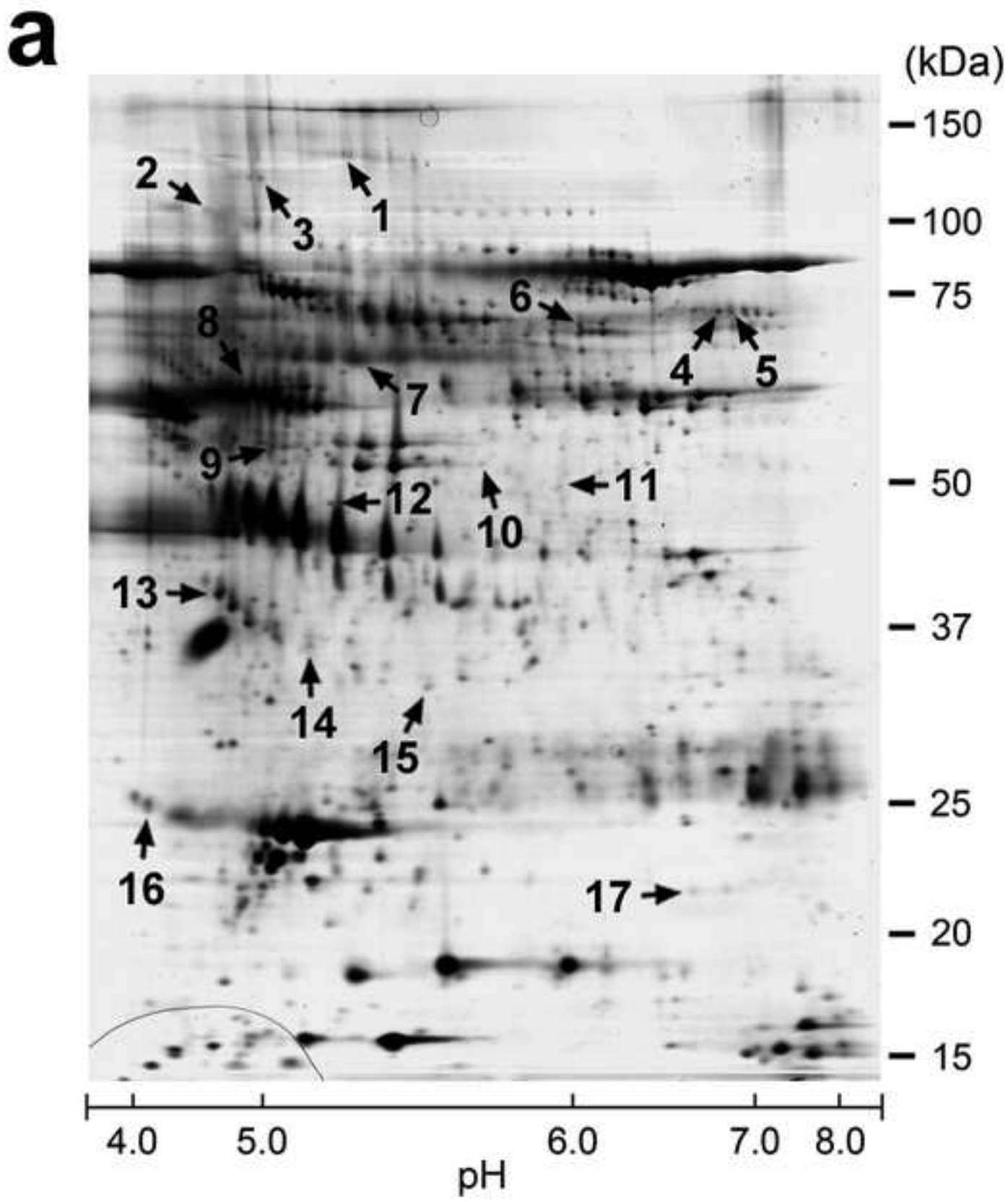

Figure 1, panel (b)

Click here to download Figure Figure, panel (b).tif

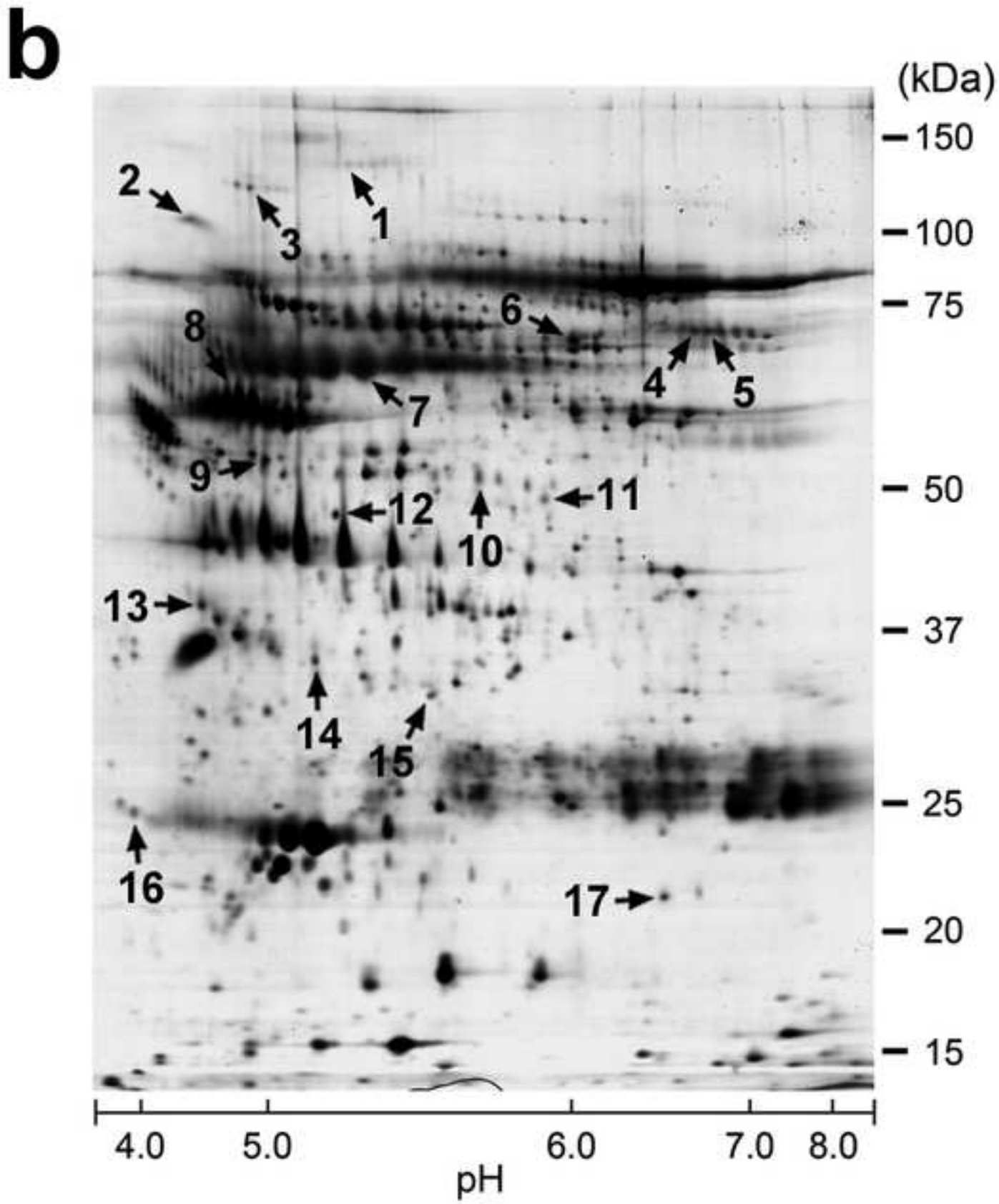

Figure 1, panel (c)

Click here to download Figure Figure, panel (c).tif

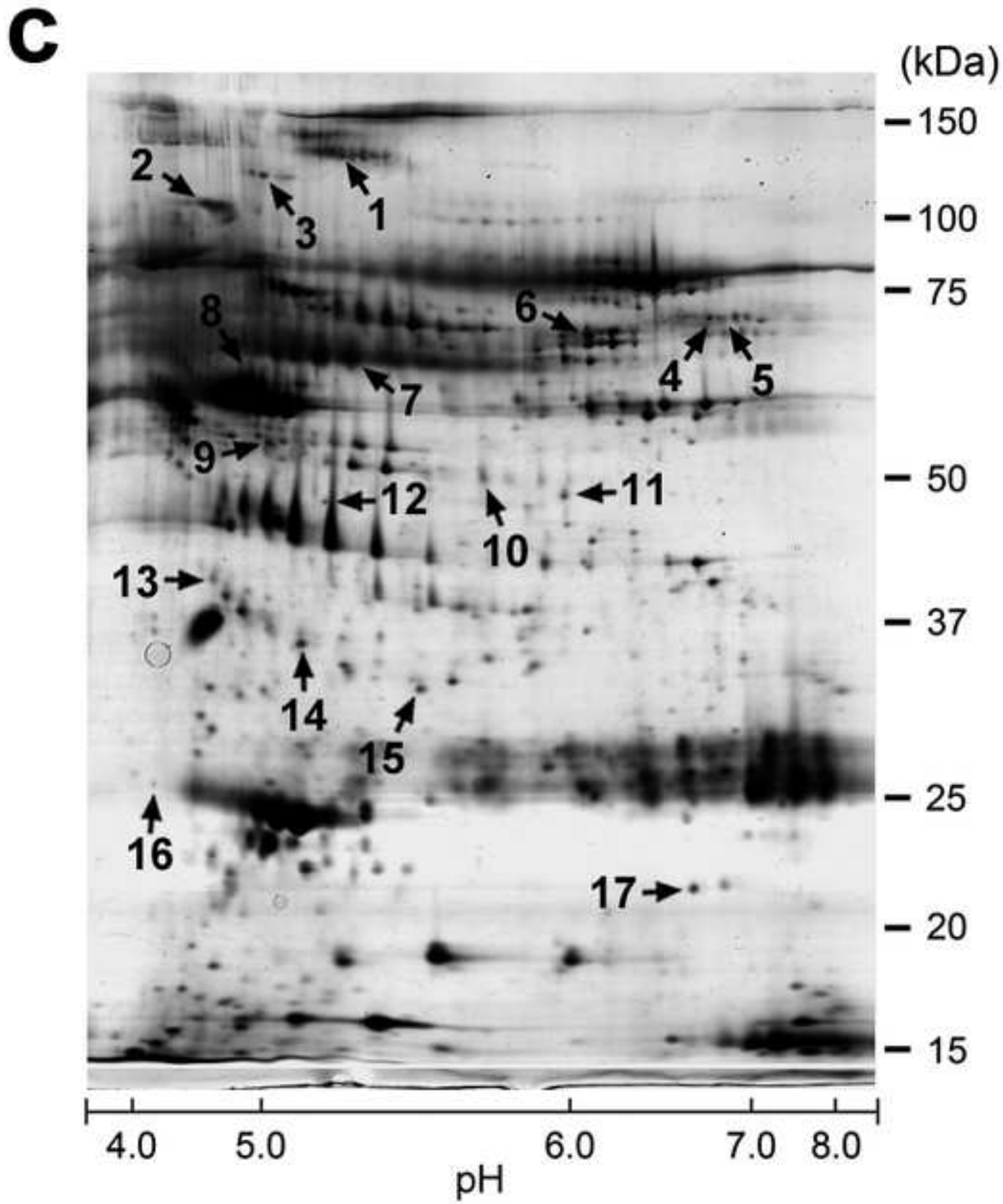

Figure 1, panel (d)

Click here to download Figure Figure, panel (d).tif

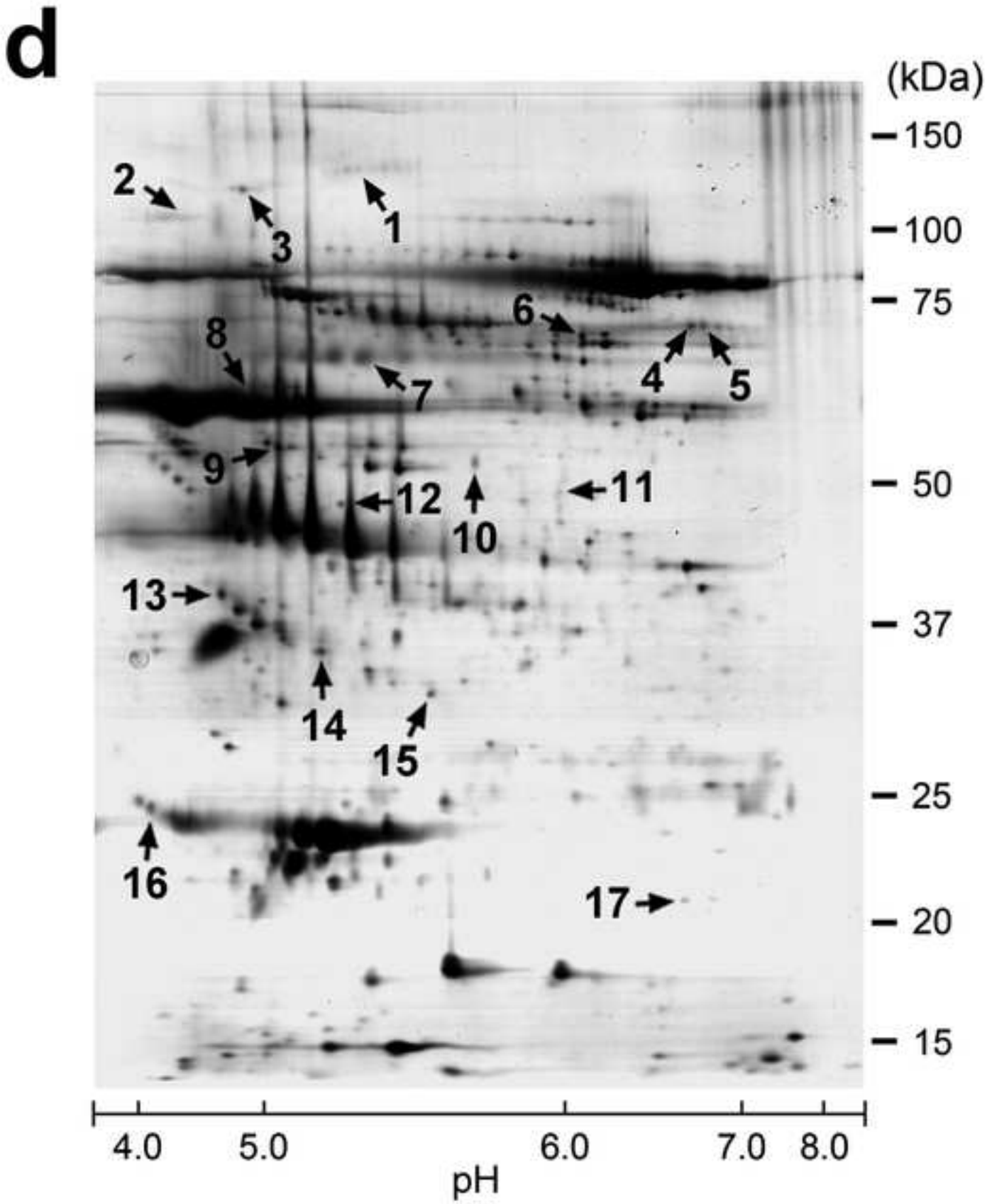

Figure 1, panel (e)

Click here to download Figure Figure, panel (e).tif

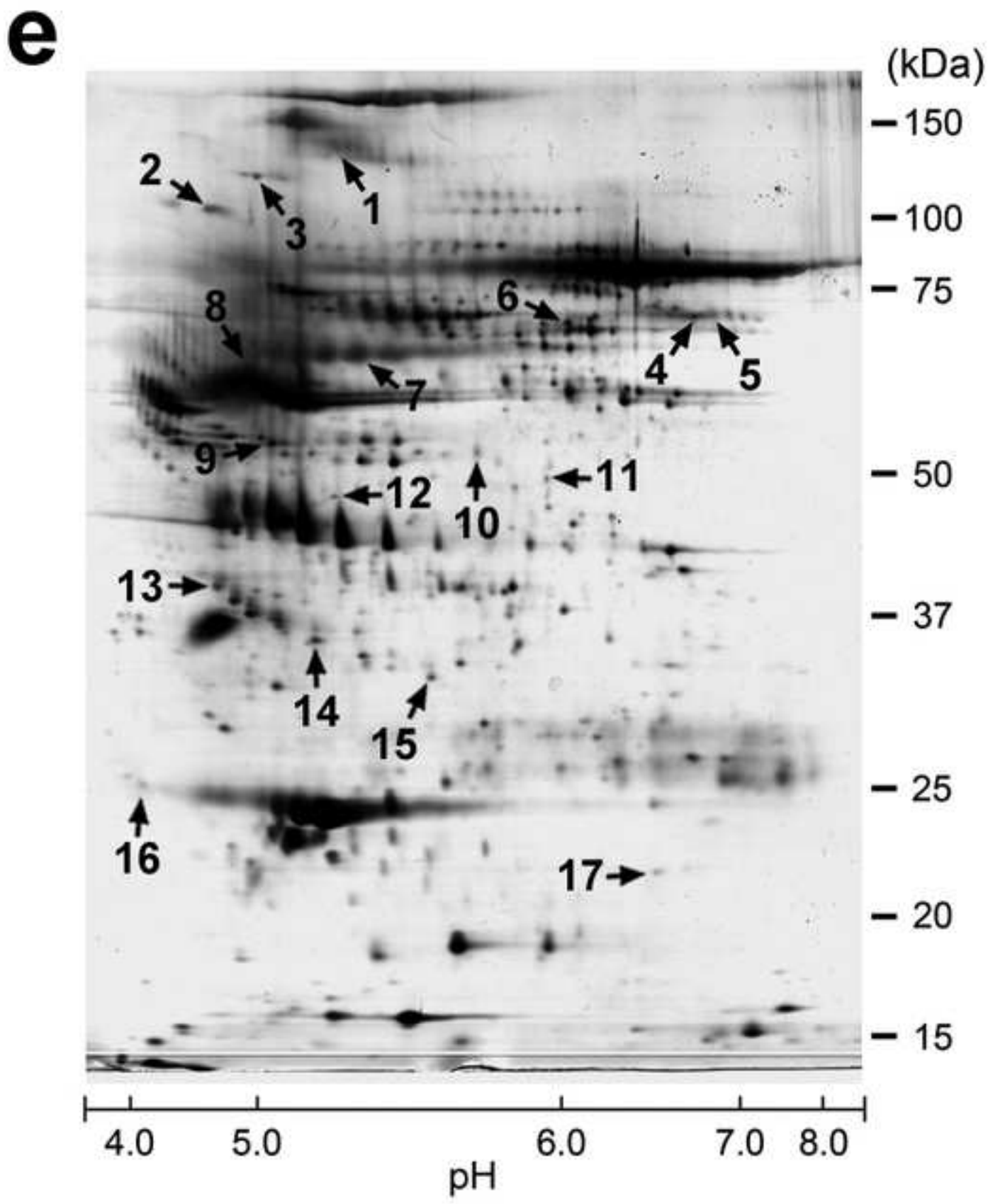

Figure 2, panel (a)

Click here to download Figure Figure 2, panel (a).tif

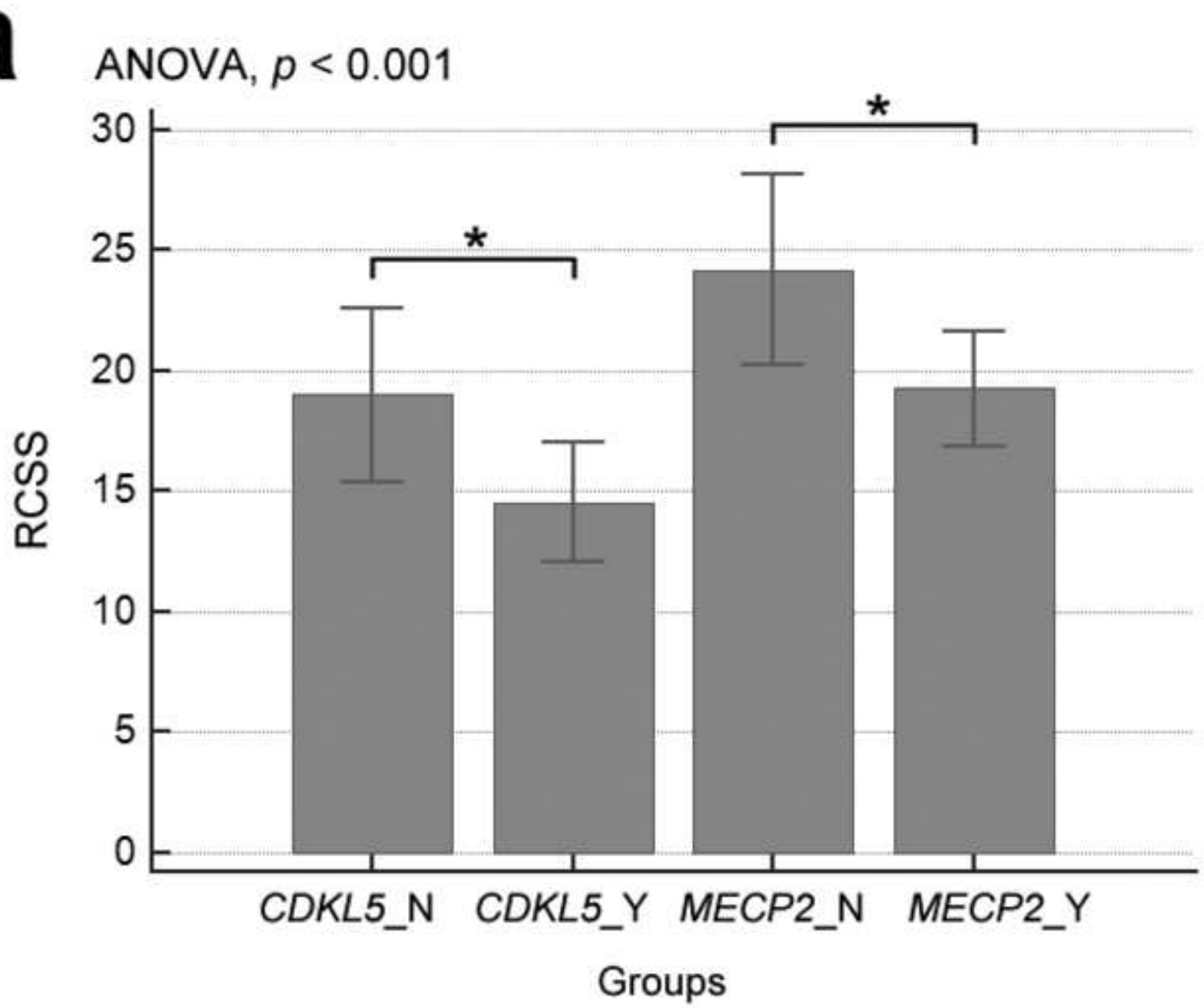

Figure 2, panel (b)

Click here to download Figure Figure 2, panel (b).tif

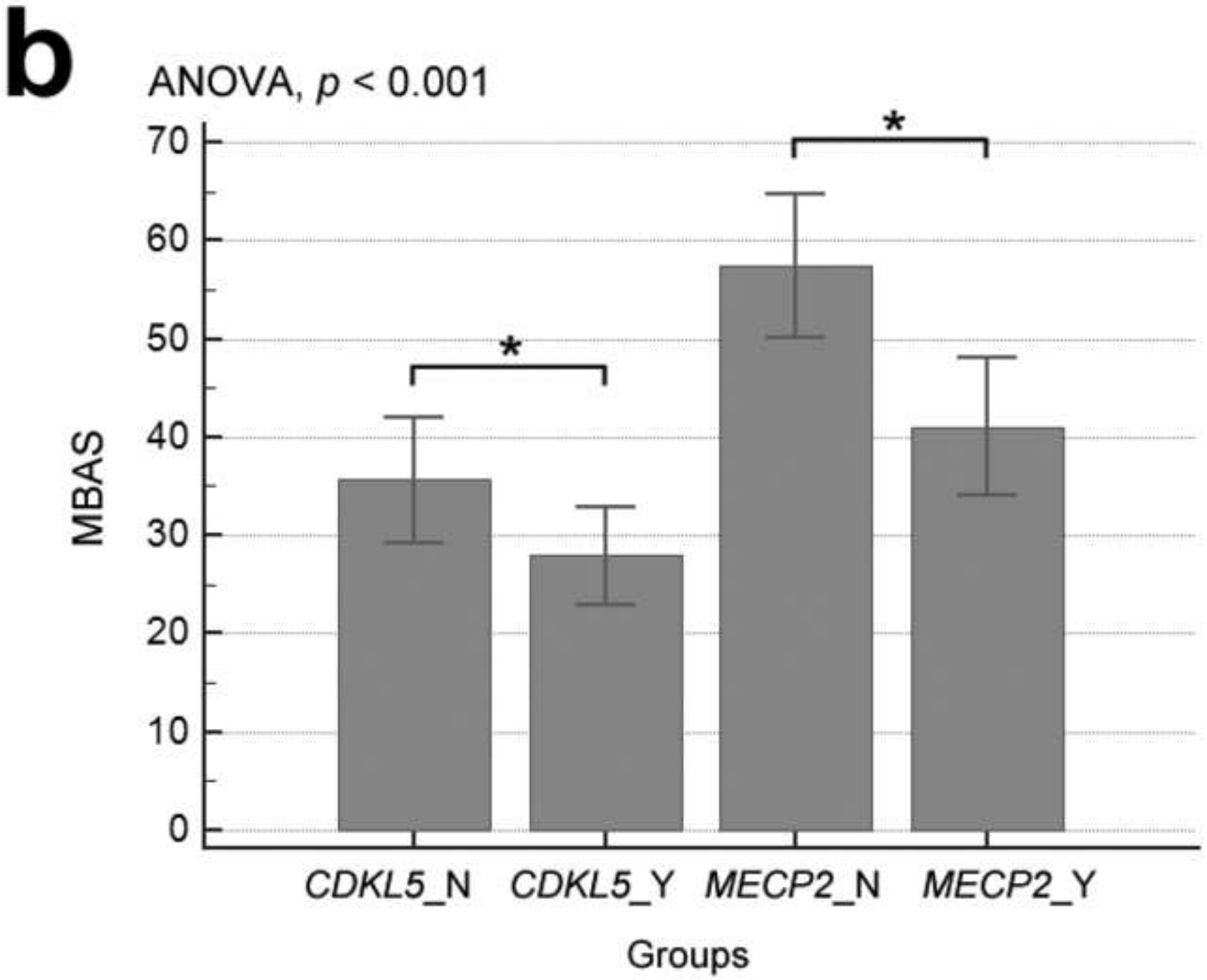

Figure 2, panel (c)

Click here to download Figure Figure 2, panel (c).tif

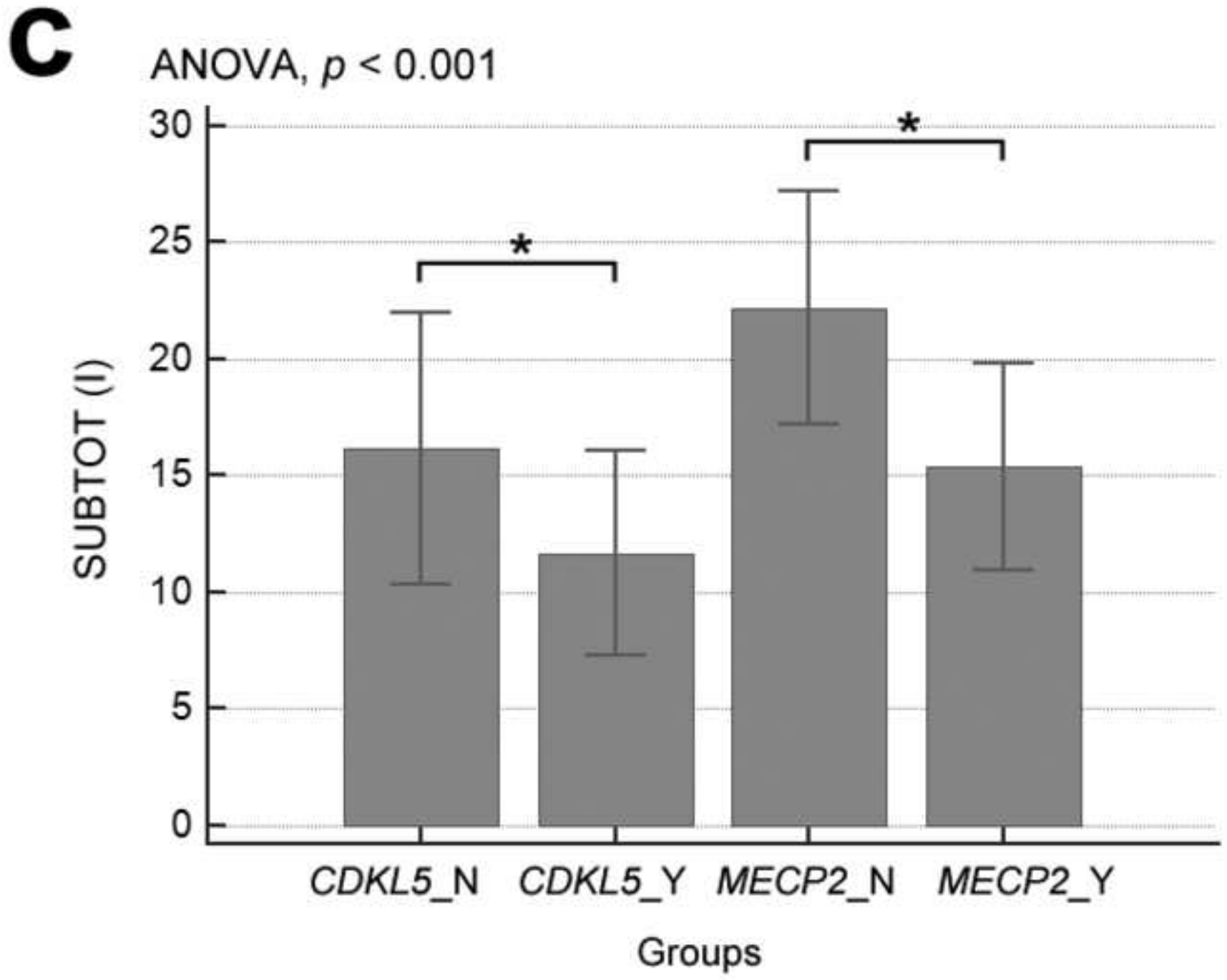

Figure 2, panel (d)

Click here to download Figure Figure 2, panel (d).tif

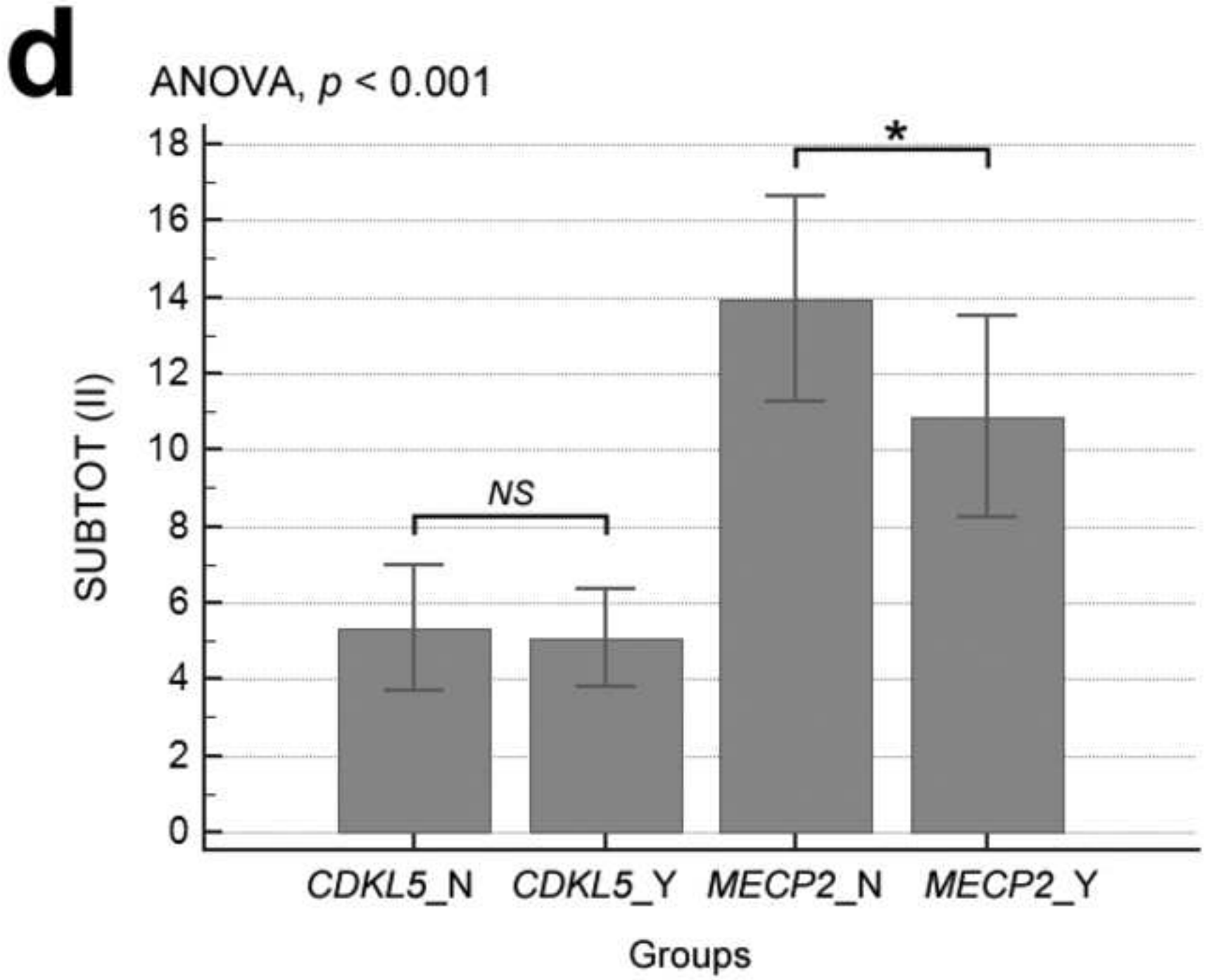

Figure 2, panel (e)

Click here to download Figure Figure 2, panel (e).tif

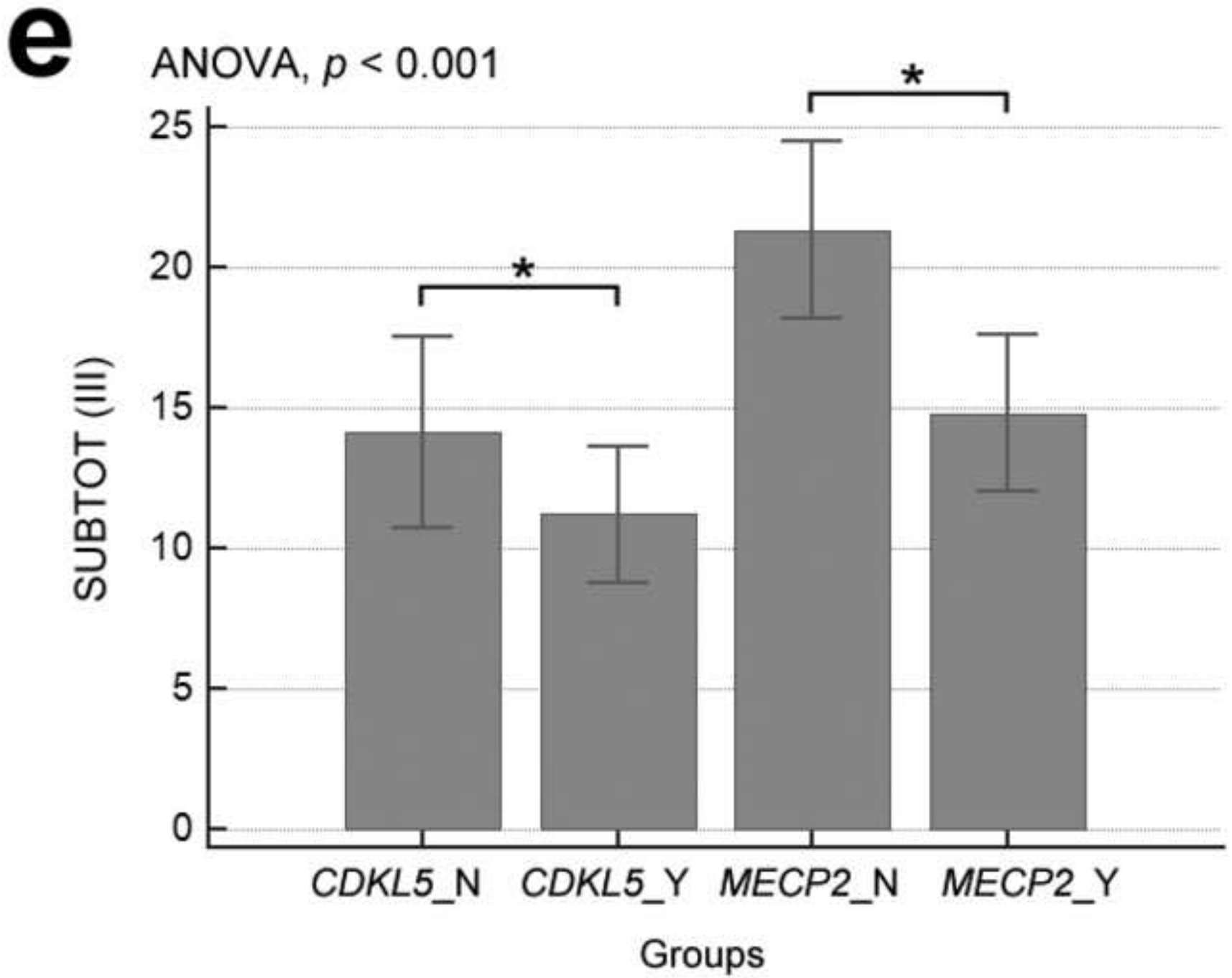

Table 1 Plasma protein variations in *CDKL5*-RTT patients: comparisons with *MECP2*-RTT patients and healthy subjects

Spot no.	Protein description	Short name	Healthy subjects	*CDKL5*-RTT	*CDKL5*-RTT *vs.* healthy subjects	*MECP2*-RTT	*MECP2*-RTT *vs.* healthy subjects	*CDKL5*-RTT *vs.* *MECP2*-RTT
1	Ceruloplasmin	CERU	1.58±0.10	1.54±0.08	=	4.12±0.11	↑**	↓**
2	Alpha-1-antitrypsin	A1AT	0.72±0.09	1.88±0.12	↑**	3.95±0.12	↑**	↓**
3	Alpha-1B-glycoprotein	A1BG	1.75±0.06	2.99±0.12	↑*	3.52±0.20	↑**	=
4	Complement C3	CO3	2.60±0.09	3.99±0.23	↑*	2.64±0.19	=	↑*
5	Complement C3	CO3	2.17±0.15	3.89±0.16	↑*	2.20±0.13	=	↑*
6	Serum transferrin	TRFE	1.79±0.12	5.90±0.10	↑**	5.98±0.09	↑**	=
7	Ig alpha-1-chain C region	IGHA1	8.25±0.21	17.29±0.30	↑**	16.53±0.31	↑**	=
8	Alpha-1-antitrypsin	A1AT	17.30±0.32	23.92±0.89	↑**	26.12±0.65	↑**	=
9	Alpha-1-antitrypsin	A1AT	1.72±0.15	3.13±0.11	↑*	3.10±0.17	↑*	=
10	Ig mu chain C region	IGHM	1.05±0.09	2.52±0.27	↑**	2.19±0.21	↑**	=
11	Serum transferrin	TRFE	0.99±0.02	1.61±0.13	↑*	2.16±0.11	↑**	=
12	CD5 antigen-like	CD5L	1.60±0.10	3.19±0.22	↑**	1.76±0.14	=	↑**
13	Clusterin	CLUS	5.05±0.23	3.01±0.09	↓*	1.80±0.11	↓**	↑**
14	Inter-alpha-trypsin inhibitor H4	ITIH4	1.73±0.09	3.11±0.14	↑*	3.20±0.12	↑*	=
15	Inter-alpha-trypsin inhibitor H4	ITIH4	1.53±0.11	3.07±0.10	↑**	3.19±0.11	↑**	=
16	Apolipoprotein A1	APOA1	4.52±0.09	3.05±0.11	↓*	1.07±0.08	↓**	↑**
17	Fibrinogen alpha chain	FIBA	0.90±0.10	2.16±0.10	↑**	2.50±0.18	↑**	=

Expression values are reported as mean ± standard deviation and labeled with (↓) decreased, (↑) increased, and (=) not detectable changes. Two-tailed *p*-values < 0.05(*) and < 0.01(**) are shown. Spot numbers refer to the protein patterns of panels (**a**), (**b**) and (**c**) from the Figure 1.

Table 2

Table 2 Circulating cytokines levels and effects of ω-3 PUFAs supplementation

Cytokines (pg/mL)	**Healthy subjects (a)**	***CDKL5*-RTT (b)**	***MECP2*-RTT (c)**	***CDKL5*-RTT after ω-3 PUFAs supplementation (d)**	***MECP2*-RTT after ω-3 PUFAs supplementation (e)**	**ANOVA *p*-value (post hoc analysis)**
TNF-α [22, 30]	0.01 [0.0-13.82]	70.73 [66.23-123.3]	97.43 [91.18-111.2]	0.01 [0.009-0.01]	6.73 [0.0-7.27]	< 0.0001 (a≠b; a≠c; b≠d; c≠e)
IL-1β [22, 30]	2.07 [0.0-2.29]	5.91 [3.26-11.56]	2.09 [1.92-2.28]	7.05 [6.23-7.19]	1.68 [0.0-2.40]	< 0.0001 (a≠b; a≠d; b≠c)
IFN-γ [22, 30]	14.06 [13.32-14.95]	25.32 [23.49-26.36]	9.12 [8.74-9.68]	25.95 [25.11-35.48]	16.49 [15.81-17.00]	< 0.0001 (a≠b; a≠d; b≠c; c≠e)
IL-12p70 [22]	11.83 [11.02-12.53]	35.50 [33.67-37.70]	0.001 [0.0-0.001]	N.A.	27.70 [27.60-27.80]	< 0.0001 (a≠b; b≠c; c≠e)
IL-10 [22, 30]	1.61 [0.0-1.89]	20.78 [19.29-25.58]	1.12 [0.0-1.25]	10.71 [10.50-12.17]	4.55 [0.0-4.60]	< 0.0001 (a≠b; a≠d; b≠c)
IL-5 [22, 30]	5.69 [5.23-6.04]	6.78 [6.32-7.05]	6.03 [5.97-6.27]	6.36 [5.63-6.73]	6.22 [6.22-6.22]	0.0003 (a≠b; a≠c; a≠d; a≠e)
IL-6 [22, 30]	2.14 [1.14-2.36]	2.18 [0.57-4.09]	5.95 [3.01-6.26]	8.91 [8.55-10.01]	9.00 [3.89-9.54]	< 0.0001 (a≠c; a≠d; a≠e; c≠e)
IL-17A [31]	7.18 [6.66-7.69]	2.07 [2.03-2.24]	20.42 [4.77-21.53]	0.01 [0.009-0.01]	8.78 [1.76-9.39]	< 0.0001 (a≠d; b≠c)
IL-22 [31]	94.74 [89.96-98.57]	108.4 [97.84-113.6]	45.86 [42.78-48.07]	N.A.	95.93 [87.21-98.84]	< 0.0001 (a≠c; b≠c; c≠e)
IL-8[#] [30]	19.86 [17.38-21.01]	25.37 [24.03-41.62]	33.87 [30.50-42.11]	0.009 [0.0-0.01]	19.88 [18.72-62.17]	< 0.0001 (a≠c; a≠d; b≠c)
RANTES[#] [30]	2.063 [1.978-2273]	1.994 [1.847-2160]	1.921 [1.758-2,013]	2.122 [2.083-2.290]	1.755 [1.585-1.840]	< 0.0001 (a≠e)
I-TAC[#] [30]	935.6 [859.2-1002]	395.0 [345.6-410.3]	434.5 [323.9-474.8]	858.2 [756.8-858.2]	912.4 [903.9-1019]	< 0.0001 (a≠b; a≠c; c≠e)
TGF-β1 [22]	30,278 [28,461-30,883]	32,886 [31,155-35,655]	27,992 [27,420-29,134]	41,962 [41,140-42,374]	29,149 [27,418-30,015]	< 0.0001 (a≠d; b≠c)
IL-37 [32]	80.00 [75.20-84.00]	192.1 [118.0-262.9]	346.3 [332.6-363.5]	255.1 [242.1-268.1]	198.8 [77.99-209.0]	< 0.0001 (a≠b; a≠c; a≠d; b≠c; c≠e)

Numbers in squared brackets in the left column refer to references cited in the main text. [#]Chemokines; N.A.: data not available; data are expressed as medians with 95% confidence intervals for median in brackets.

Table 3 Correlation matrix between differentially expressed proteins and circulating cytokines

Cytokines (Inflamm. action)[§]	**Plasma proteins** (+, positive APR proteins; -, negative APR proteins)																
	(+) CERU (spot #1)	(+) A1AT (spot #2)	(+) A1BG (spot #3)	(+) CO3 (spot #4)	(+) CO3 (spot #5)	(-) TRFE (spot #6)	N.A. IGHA1 (spot #7)	(+) A1AT (spot #8)	(+) A1AT (spot #9)	N.A. IGHM (spot #10)	(-) TRFE (spot #11)	N.A. CD5L (spot #12)	N.A. CLUS (spot #13)	(+) ITIH4 (spot #14)	(+) ITIH4 (spot #15)	(-) APOA1 (spot #16)	(+) FIBA (spot #17)
TNF-α (+)	**0.282***	**0.546****	**0.704****	**0.240***	0.036	**0.596****	**0.637****	**0.644****	**0.354****	**0.627****	**0.596****	0.139	**-0.538****	**0.651****	**0.672****	**-0.405****	**0.661****
IL-1β (+)	**-0.506****	-0.168	0.038	**0.382****	**0.665****	0.086	0.126	0.018	**0.395****	**0.406****	-0.191	**0.702****	0.108	0.124	0.207	0.222	0.111
IFN-γ (+)	**-0.667****	**-0.309****	**-0.234***	**0.286****	**0.590****	-0.165	-0.117	**-0.246***	0.190	0.093	**-0.369****	**0.482****	**0.331****	-0.097	-0.116	**0.228***	-0.203
IL-12p70 (+)	-0.162	0.022	0.076	**0.447****	0.210	0.108	**0.454****	0.095	-0.012	**0.243***	0.095	0.104	0.021	0.167	0.057	-0.118	0.011
IL-10 (-)	**-0.615****	-0.204	0.042	**0.426****	**0.561****	0.118	0.206	0.008	**0.323****	**0.353****	-0.122	**0.480****	0.100	0.154	0.130	0.152	0.086
IL-5 (-/+)	0.052	-0.214	**0.278***	0.066	0.039	**0.334****	**0.272***	**0.335****	**0.473****	**0.343****	**0.238***	0.088	**-0.386****	**0.437****	**0.367****	**-0.286***	**0.305****
IL-6 (+/-)	**0.2621***	**0.269***	0.098	-0.168	-0.128	0.072	-0.181	0.092	0.188	0.043	0.137	-0.097	**-0.276***	0.018	0.074	**-0.344****	0.109
IL-17A (+)	**0.586****	**0.411****	**0.292***	-0.046	**-0.330****	0.135	0.190	**0.262***	**-0.237***	0.081	**0.416****	**-0.322****	**-0.392****	0.177	0.173	**-0.398****	0.217
IL-22 (-/+)	-0.186	-0.089	-0.008	**0.424****	0.148	0.044	0.186	-0.049	**-0.319****	0.067	0.093	0.166	0.216	-0.109	-0.007	0.005	-0.042
IL-8 (+)	**0.474****	**0.488****	**0.533****	0.208	-0.021	**0.430****	**0.512****	**0.535****	0.102	**0.484****	**0.520****	-0.119	**-0.444****	**0.484****	**0.485****	**-0.460****	**0.445****
RANTES (+)	**-0.298****	**-0.488****	**-0.276***	-0.111	0.183	**-0.287***	-0.203	**-0.290***	0.018	-0.071	**-0.381****	0.217	**0.394****	-0.210	-0.209	**0.513****	-0.158
I-TAC (+)	0.037	**-0.331****	**-0.617****	**-0.452****	**-0.391****	**-0.602****	**-0.573****	**-0.546****	**-0.569****	**-0.668****	**-0.382****	**-0.600****	**0.292***	**-0.662****	**-0.625****	0.192	**-0.620****
TGF-β1 (-)	**-0.630****	**-0.432****	**-0.250***	0.145	**0.580****	-0.166	-0.147	**-0.281***	0.176	0.065	**-0.404****	**0.595****	**0.362****	-0.147	-0.102	**0.437****	-0.157
IL-37 (-)	**0.275***	**0.564****	**0.590****	0.0209	-0.022	**0.620****	**0.315****	**0.617****	**0.524****	**0.506****	**0.516****	0.194	**-0.599****	**0.532****	**0.638****	**-0.397****	**0.691****

Correlations are expressed as Spearman's rank *Rho* coefficients, with the exceptions of IL-5 and IL-22 where the values are Pearson's *r* coefficients. Significant correlations are highlighted in bold. Two-tailed *p*-values < 0.05(*) and < 0.01(**) are shown. N.A., not applicable. Spot numbers refer to those reported in Figure 1 and Table 1. [§]Inflammatory action based on literature (+: pro-inflammatory; -: anti-inflammatory; +/- or -/+: mixed action). Relationships between plasma proteins and cytokines were considered consistent in the occurrence of positive APR proteins *vs.* pro-inflammatory cytokines or negative APR proteins *vs.* anti-inflammatory cytokines, or positive APR proteins *vs.* inflammation antagonizing cytokines such as IL-37, which mirror the positive APR proteins behaviour.

Table 4 Modulatory effects of ω-3 PUFAs on inflammatory protein patterns in *CDKL5*-RTT and *MECP2*-RTT

Spot no.	Protein description	Short name	*CDKL5*-RTT after ω-3 PUFAs supplementation	Treated *CDKL5*-RTT *vs.* untreated *CDKL5*-RTT	*MECP2*-RTT after ω-3 PUFAs supplementation	Treated *MECP2*-RTT *vs.* untreated *MECP2*-RTT
1	Ceruloplasmin	CERU	1.51±0.09	=	2.16±0.19	↓*P
2	Alpha-1-antitrypsin	A1AT	1.10±0.10	↓*P	2.11±0.14	↓*P
3	Alpha-1B-glycoprotein	A1BG	2.03±0.10	↓*C	2.15±0.12	↓*P
4	Complement C3	CO3	2.64±0.09	↓*C	2.73±0.20	=
5	Complement C3	CO3	2.77±0.10	↓*C	2.19±0.11	=
6	Serum transferrin	TRFE	2.95±0.11	↓*P	3.08±0.10	↓*P
7	Ig alpha-1-chain C region	IGHA1	8.27±0.17	↓**C	8.65±0.35	↓**C
8	Alpha-1-antitrypsin	A1AT	20.05±0.33	↓*P	21.22±0.55	↓*P
9	Alpha-1-antitrypsin	A1AT	3.08±0.12	=	3.04±0.07	=
10	Ig mu chain C region	IGHM	1.53±0.16	↓*P	1.46±0.11	↓*P
11	Serum transferrin	TRFE	1.08±0.09	↓*C	1.55±0.08	↓*P
12	CD5 antigen-like	CD5L	2.04±0.12	↓*P	1.64±0.12	=
13	Clusterin	CLUS	3.50±0.27	=	2.96±0.08	↑*P
14	Inter-alpha-trypsin inhibitor H4	ITIH4	2.55±0.11	=	2.70±0.13	=
15	Inter-alpha-trypsin inhibitor H4	ITIH4	2.08±0.08	↓*P	2.15±0.10	↓*P
16	Apolipoprotein A1	APOA1	3.53±0.28	=	1.08±0.09	=
17	Fibrinogen alpha chain	FIBA	1.33±0.13	↓*P	1.40±0.11	↓*P

P: proteins partially rescued after ω-3 PUFAs supplementation; C: completely rescued after ω-3 PUFAs supplementation; expression values are expressed as mean ± standard deviation and labeled with (↓) decreased, (↑) increased, and (=) not detectable changes. Two-tailed *p*-values < 0.05(*) and < 0.01(**) are shown. Spot numbers refer to the protein patterns of panels (**d**) and (**e**) from the Figure 1.

Supplementary Table 2

Supplementary Table 4