Ectodysplasin A in biological fluids and diagnosis of ectodermal dysplasia

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ABSTRACT

The TNF family ligand ectodysplasin A (EDA) is produced as two full-length splice variants, EDA1 and EDA2, that bind to EDA receptor (EDAR) and X-linked EDA receptor (XEDAR/EDA2R) respectively. Inactivating mutations in the genes *EDA* or *EDAR* genes cause hypohidrotic ectodermal dysplasia (HED) with malformation of teeth, hair and glands, while milder deficiencies affect teeth only. EDA acts early during development of ectodermal appendages as early as the embryonic placode stage, and also plays a role in adult appendage function. We detected three- to fourfold higher levels of circulating EDA in cord blood than in adult sera. A receptor binding-competent form of EDA1 was the main form of EDA, but a minor fraction of EDA2 was also found in fetal bovine serum. Sera of EDA-deficient patients contained either background EDA levels or low levels of EDA that could not bind to recombinant EDAR. Serum of a patient with a V262F missense mutation in *EDA* causing a milder form of X-linked HED (XLHED) contained low levels of EDA capable of binding to EDAR. In two mildly affected carriers, intermediate levels of EDA were detected, while a severely affected carrier had no active EDA in serum. Small amounts of EDA were also detectable in normal adult saliva. Finally, EDA could be measured in spots of wild-type adult or cord blood dried onto filter paper at levels significantly higher than in EDA-deficient blood. Measurement of EDA levels combined with a receptor-binding assay might be of relevance to aid diagnosis of total or partial EDA deficiencies.

INTRODUCTION

EDA is a TNF family member important for the development of ectodermal appendages in vertebrates (Harris et al. 2008; Kere et al. 1996; Monreal et al. 1998; Srivastava et al. 1997). EDA-deficiency in humans causes X-linked hypohidrotic ectodermal dysplasia (XLHED), a congenital disorder characterized by hypoplastic hair, teeth and sweat glands (Visinoni et al. 2009). The EDA protein consists of a short intracellular domain, a membrane spanning segment, a stalk region, a consensus furin cleavage site, a short proteoglycan-interacting sequence, an oligomerizing collagen domain and a C-terminal TNF homology domain that forms homotrimers and binds to receptors (Chen et al. 2001; Schneider et al. 2001a; Swee et al. 2009). Two EDA splice variants differing by only two amino acids (Yan et al. 2000) bind to distinct receptors: EDA1 binds to EDA receptor (EDAR), and EDA2 binds to X-linked EDAR (XEDAR/EDA2R) (Tucker et al. 2000; Yan et al. 2000). EDA1-EDAR interactions mediate development of ectodermal appendages, while EDA2-XEDAR may serve as downstream effectors of the p53-induced anti-proliferative response in colon cancer (Tanikawa et al. 2010). Fully or partially inactivating mutations of the gene *EDA* provoke XLHED and non-syndromic tooth agenesis, respectively (Mues et al. 2010). Remarkably, human, dog, cow and mouse EDA are 100% identical in their 145 amino acid-long receptor-binding domains, 98% identical to chicken EDA and 63% identical (82% similar) to a fish EDA (*Gasterosteus acuelatus*). During hair placode formation in mice, EDA is one of the most apical signaling molecules downstream of Wnt. EDA is in turn essential, via NF-κB activation, for sustained Wnt activity (Zhang et al. 2009). Primary hair placodes do not form in *Eda*-deficient mice, teeth are present but small and abnormally shaped, sweat glands are missing, and a number of other glands are either absent or reduced (Gruneberg 1971).

Transgenic expression of EDA1 in the skin of wild-type mice induced supernumerary tooth and mammary gland formation, longer hair and nails, and enlarged sebaceous glands (Mustonen et al. 2003). In *Eda*-deficient mice, the transgene stimulated formation of guard hairs, sweat glands and induced sebaceous glands hypertrophy (Cui et al. 2003). Repression of transgenic EDA1 in adult mice normalized sebaceous glands, indicating a limiting role of EDA1 also in some adult structures (Cui et al. 2003). The prenatal or perinatal, but not adult, administration of recombinant EDA1 or of agonist anti-EDAR antibodies to *Eda*-deficient mice or dogs ameliorated the EDA-deficient phenotype, paving the way for protein replacement therapy in XLHED (Casal et al. 2007; Gaide and Schneider 2003; Kowalczyk et al. 2011). Thus, early diagnosis of EDA-deficiency in humans is warranted, particularly since an EDA replacement drug has been under investigation for use in newborn infants with XLHED (www.clinicaltrials.gov NCT01775462 and NCT01992289). However, early diagnosis of XLHED is not trivial. If family history suggests a risk, and if this is known to the doctor, diagnosis can sometimes be established prenatally when ultrasound examination reveals a significant deficit of tooth germ formation (Wunsche et al. 2015), or at birth when an array of clinical features such as absence of sweat gland openings in the epidermis, skin dryness or specific craniofacial abnormalities can be observed. Sometimes, diagnosis is only made in the second year of life upon failure of tooth eruption. Definitive evidence of XLHED as opposed to other forms of ectodermal dysplasia or tooth agenesis is provided by identification of the causative mutation in *EDA* (Burger et al. 2014; Schneider et al. 2011).

In this study, we measured EDA in serum, saliva and dried blood spots. We report the detection of circulating EDA that is capable of binding to receptors, contains the protein's collagen domain and displays a predominance of the EDA1 over the EDA2 isoform. EDA levels

were high in fetuses and newborn, lower in adults, and very low to absent in XLHED patients, in line with the known roles of EDA during development, adulthood and disease. These results indicate the possibility of diagnosing XLHED by measuring EDA and its receptor-binding ability in biological fluids.

MATERIAL AND METHODS

Animals – K14-*Eda-A1* transgenic mice, *Edar*-deficient OVE1B mice, *Eda*-deficient *Tabby* mice and their wild-type controls were as described (Kowalczyk et al. 2011; Kowalczyk-Quintas et al. 2014b; Mustonen et al. 2003). Mice were handled according to guidelines and under the authorization of the Swiss Federal Food Safety and Veterinary Office (authorization 1370.6 to PS) or in accordance with the guidelines and with approval from the Finnish National Board of Animal Experimentation.

Human samples – Unstimulated saliva and serum samples were obtained from adult patients affected by XLHED, carriers of *EDA* mutations or non-affected controls (age range: 21 to 52 years-old for all groups). Sera were also prepared from cord blood of neonates of from cord blood of pre-term babies. In some cases, blood was applied directly onto filter paper cards for blood sampling (Perkin-Elmer). Samples were obtained with informed consent at the University Hospital of Erlangen. All samples were stored at -70°C. Analyses were performed in Lausanne under the approval of the Commission cantonale d'éthique de la recherche sur l'être humain, Lausanne.

Antibodies, recombinant proteins and plasmids – Anti-EDA antibodies EctoD2 and biotinylated EctoD3, EDAR-Fc, XEDAR-Fc and BMCA-Fc were as previously described (Kowalczyk-Quintas et al. 2014b; Schneider et al. 2001a; Schneider et al. 2001b). Fc-EDA1

(EDI200) was provided by Edimer Pharmaceuticals. Anti-EDA monoclonal antibody Renzo-2 is commercially available (Enzo Life Sciences, ALX-522-038). Sequences of proteins encoded by plasmids used for this study are listed in appendix Table 1.

Affinity purification of EDA –EctoD2 and EctoD3 were coupled at 5 mg/ml to NHS-activated Sepharose (GE Healthcare), used to capture EDA in 500 ml of fetal calf serum, washed extensively with PBS and eluted with 50 mM citrate-NaOH pH 2.7. The eluate was neutralized with 1 M Tris-HCl pH9, concentrated and buffer was exchanged for PBS with a 30 kDa cutoff centrifugal concentrator device.

Deglycosylation – Denatured EDA samples were digested with peptide N-glycanase F according to manufacturer's instructions (NewEngland Biolabs).

Immunoprecipitations and Western blot – EDA was immuno-precipitated for 16 h at 4°C with 10 µl of Protein A-Sepharose beads and 1 µg of EDAR-Fc or of XEDAR-Fc in 500 µl of PBS. Beads were collected in mini columns (Schneider et al. 2014), washed with PBS, and eluted with 15 µl of 100 mM citrate-NaOH pH 3. The eluate was neutralized with 5 µl of 1 M Tris-HCl pH9. EDA was also incubated for 16 h at 4°C with 10 µl of heparin-Sepharose beads in 500 µl of PBS. Beads were washed with PBS and eluted with PBS, 0.8 M NaCl. Western blot was performed according to standard protocols with samples heated in denaturing and reducing conditions for 5 min at 70°C, because EDA tends to aggregate when boiled. Western blots were revealed with Renzo-2 at 1 µg/ml, followed by horseradish peroxidase-coupled anti-mouse secondary reagent and ECL. When required, the membrane was probed again with horseradish peroxidase-coupled anti-human IgG.

AlphaLISA – Five µl of samples (*e.g.* serum diluted 1/2 or undiluted saliva with or without pre-depletion on ELISA plates coated with BCMA-Fc, EDAR-Fc or EctoD2) were mixed with

biotinylated EctoD3 at 15 ng/ml and 0.5 µg of EctoD2 acceptor beads, and signal was recorded with an Enspire plate reader (Perkin-Elmer). Standard curves were generated with Fc-EDA1 diluted into buffer or into XLHED serum or saliva as indicated. For the measurement of EDA in dry blood spots, blood was eluted from filters papers in PBS, then immunoprecipitated with 0.2 µl of EctoD2 acceptor beads for 1 h at room temperature prior to measurement by AlphaLISA. Further details are available in the online supplemental appendix.

Gel permeation chromatography – 300 µl of serum from human cord blood with high EDA content, or from normal human serum with low EDA content, were loaded onto a Superdex 200 column equilibrated and eluted in PBS. 1 ml fractions were collected. EDA was measured in these fractions by AlphaLISA.

Statistics – Statistics were performed by one-way analysis of variance with Bonferroni's multiple comparison tests using Prism. P values lower than 0.05 were considered significant.

RESULTS

Specific detection of EDA in serum reveals higher circulating EDA levels in the fetal and newborn stages compared to adult – ELISA experiments using a previously characterized pair of mouse IgG1 anti-EDA antibodies (EctoD2 and EctoD3) (Kowalczyk-Quintas et al. 2014b) indicated the feasibility of detecting endogenous EDA in adult serum (appendix Fig. 1). When used in a homogenous AlphaLISA assay (Eglen et al. 2008), this pair of antibodies detected a standard of Fc-EDA1 diluted in EDA-deficient serum with a sensitivity of about 0.1 ng/ml in as little as 2.5 µl of serum. Signals obtained in a panel of XLHED sera were consistently very low (Fig. 1A,B). In this cohort of XLHED patients, the majority is not expected to produce any soluble EDA protein as a consequence of frameshift mutation, exon deletion or duplication,

splice site mutation or mutations affecting the furin cleavage site (Table 1). In two patients with point mutations in the extracellular domain that may preserve the EDA protein, EDA levels were higher (0.15 ng/ml) and similar to those found in three carriers, one of which was severely affected by XLHED. These levels remained lower than the average EDA level in adult sera (0.7 ng/ml) or in cord blood sera (2.5 ng/ml) (Fig. 1B). We found no gender differences of EDA levels in wild-type humans, neither at birth nor in adults. EDA levels reached 4 ng/ml in cord blood sera of two pre-term babies, similar to what was measured in fetal calf serum. EDA was present at only 1.5 ng/ml in a third pre-term baby whose pre-term delivery was due to a generalized infection. Taken together, these results indicate that circulating EDA levels are significantly higher in premature and newborn babies than in adults, and very low or at background in EDA-deficient XLHED patients (Fig. 2C).

Low levels of circulating EDA in mice – EDA levels in wild-type adult mouse serum were low (0.13 ng/ml), but higher than those in an EDA-deficient serum. Curiously, circulating EDA levels in EDAR-deficient or in EDA1-transgenic mice, where the transgene is expressed in the skin under a keratin-14 promoter, were not different from wild-type (Fig. 1D).

A fraction of circulating wild-type EDA is capable of binding to EDAR – Antibodies used in this study recognize and inhibit native forms of both EDA1 and EDA2 and therefore cannot distinguish between the protein products of the two splice variants (Kowalczyk-Quintas et al. 2014b). EDAR however specifically binds to EDA1, as does its recombinant protein EDAR-Fc (Schneider et al. 2001a; Yan et al. 2000). Using a set of three different pre-depletions, including one on EDAR-Fc, it was possible to estimate that about three quarters (52 - 94%) of wild-type

EDA in the circulation can bind to EDAR. We have not been able to further characterize the fraction of EDA that does not bind to EDAR-Fc: this could be inactive EDA, EDA in complex with endogenous soluble receptors or heteromers of EDA1 and EDA2. Pre-depletion with EDAR-Fc was also successful in two carriers and in a patient with the point mutation V262F (Fig. 2A-C). The later XLHED patient was rather mildly affected, with oligodontia and moderately reduced ability to sweat. Binding to EDAR was however defective in a severely affected carrier, in a patient with the point mutation M364R and in a few XLHED patients with EDA levels slightly above background, suggesting that these circulating EDA molecules are not functional (Fig. 2B,C). The patient with the mutation M364R is severely affected, with only 5 teeth and very little ability to sweat. In conclusion, the quantification of serum EDA protein concentration is in itself a valuable biomarker of EDA function, but combined with the measurement of EDA receptor-binding activity can further distinguish between hypomorphic and fully inactivating mutations.

Detection of EDA in saliva and in dry blood spots – Low levels of EDA, which could be depleted on EDAR-Fc, were detected in wild-type but not in XLHED adult saliva (appendix Fig. 2). Interestingly, signals obtained with recombinant Fc-EDA1 were 4- to 5-fold lower in EDA-deficient serum compared to saliva or assay buffer (Fig. 2D). Thus, as exemplified for fetal calf serum, EDA in serum is underestimated about 5-fold (Appendix Fig. 2B) unless the standard curve is also measured in (EDA-deficient) serum (Fig. 1A). Moreover, EDA could be detected in dried blood samples. For this purpose, dry blood was eluted from filter paper in PBS, then immunoprecipitated with EctoD2-coupled AlphaLISA acceptor beads to circumvent the quenching effect of concentrated hemoglobin, and measured for EDA by AlphaLISA. In this

procedure, the yield of Fc-EDA immunoprecipitation was about 50%, recovery of dry Fc-EDA from filter paper was about a third, and the combined yield of both procedures approached 20% (appendix Fig. 2). With this protocol, signals were obtained in wild-type adult blood and wild-type cord blood eluted and immuno-precipitated from 6-mm diameter filter paper punches, while only background signals were found with an adult XLHED blood processed in parallel (Fig. 3C). These measures repeated at different time points after blood collection always showed higher EDA signals in wild-type adult or cord blood than in the control XLHED blood (R156QfsX2) from 3 days to more than a month after blood collection (Fig. 3C). EDA signals detected in cord blood were sometimes, but not always, higher than those of adult blood. The qualitative nature of these results perhaps reflects a sub-optimally normalized procedure. In conclusion, these results demonstrate the feasibility of measuring EDA in dry blood spots and in saliva.

Circulating bovine EDA is N-glycosylated, contains the collagen domain and exists predominantly as the EDA1 isoform with a minority of the EDA2 isoform – Circulating endogenous EDA was affinity-purified from fetal calf serum on anti-EDA antibodies, then immuno-precipitated with recombinant EDAR-Fc or XEDAR-Fc. Flag-tagged forms of EDA1 and EDA2 used as controls bound to EDAR-Fc and XEDAR-Fc, respectively (Fig. 4A). Endogenous EDA from two independent EDA preparations precipitated preferentially with EDAR-Fc and to a lesser extent with XEDAR-Fc (Fig 4A). Endogenous EDA migrated with a molecular weight of about 35 kDa under denaturing conditions, larger than Flag-EDA1, which contains only the TNF homology domain, but at the size of transfected, naturally processed full-length EDA1, suggesting that both the collagen and TNF homology domains are present in circulating EDA. Deglycosylation with peptide N-glycanase F reduced the size of endogenous

EDA from 35 to 29 kDa, and that of a minor fragment of 22 kDa to 18 kDa (Fig. 4B). This smaller fragment probably corresponds to the TNF homology domain alone. Recombinant EDA is not fully N-glycosylated, generating a characteristic doublet in the Western blot (Fig. 4A) (Schneider et al. 2001a), while endogenous EDA is almost fully N-glycosylated. The absence of intermediate glycosylation products between the 22 and 18 kDa bands in partially digested EDA further suggests that endogenous EDA, like transfected EDA expressed in cultured cells, carries a single N-linked glycan (Fig. 4B).

Endogenous EDA and naturally cleaved EDA both bound to heparin-Sepharose, while a mutant of the proteoglycan-binding domain hardly did so (Fig. 4C). Flag-tagged EDA1 and EDA2 lacking the proteoglycan-binding domain did not bind to heparin-Sepharose either (Fig. 4C). These data suggest that endogenous EDA has a functional proteoglycan-binding domain. Finally, the native size of human EDA in cord blood serum was estimated by size exclusion chromatography to be ~200 kDa (Fig. 4D). A calculated size ratio of 5.7 between native and denatured EDA suggests that EDA circulates in blood as a hexamer or, if the collagen domain confers to the protein a rod-like shape, as a non-globular trimer. In summary, endogenous bovine EDA is a soluble glycoprotein containing the proteoglycan-binding, collagen and TNF homology domains with a predominance of the EDA1 over the EDA2 isoform. Its posttranslational modifications appear to be more homogeneous that those of transfected EDA expressed in cultured cell lines.

DISCUSSION

In this study, high levels of EDA were detected in sera of fetuses and newborns, in good agreement with the known timing of EDA function in the development of ectodermal

appendages (Mikkola 2008). Lower levels of EDA were also found in adult sera and only background levels in XLHED sera. The size of endogenous EDA under native and denaturing conditions, its glycosylation and receptor binding patterns correspond to what we know about the protein (Kowalczyk-Quintas and Schneider 2014).

Expression of *EDA* mRNA has been reported in various adult human tissues (Montonen et al. 1998) and in adult mice EDA and EDAR have been implicated in regulation of the hair cycle (Fessing et al. 2006). In addition, hair-associated sebaceous glands responded to EDAR stimulation, suggesting that sebaceous glands are likely targets of EDA in the adult (Kowalczyk-Quintas et al. 2014a). Other tissue candidates for the action of EDA in adults are salivary glands (Hill et al. 2014) and wounded skin (Garcin et al. 2016) that both respond to EDAR agonists. It therefore makes sense to find EDA expressed in adults.

The measurement of EDA combined with pre-depletion on recombinant receptors showed that the main circulating isoform is EDA1, but that lower levels of EDA2 are also present in fetal calf serum. In principle, depletion of sera with recombinant XEDAR should give direct information on the proportion of circulating EDA2, but in practice the reduction observed was too low for reliable interpretation. The co-existence of EDA1 and EDA2 in serum is interesting in terms of possible heteromer formation. In the TNF family, heteromers of lymphotoxin-α/lymphotoxin-β as well as heteromers of BAFF/APRIL, whose receptor binding specificities can be distinct from those of homotrimers, have been structurally characterized (Schuepbach-Mallepell et al. 2015; Sudhamsu et al. 2013). It will be interesting in the future to investigate whether EDA1/EDA2 heteromers can be produced, and, if so, what receptor(s) they bind.

In the adult, EDA expression has been described in skin epithelium, hair follicles and teeth, but also in organs where EDA has no known functions such as the central nervous system,

kidney or prostate (Montonen et al. 1998). The origin of circulating EDA reported here in three different species remains to be determined. It may not originate exclusively from the skin because mice overexpressing EDA1 in the epidermis, where it induces pronounced morphological alterations in various appendages (Mustonen et al. 2003), had normal levels of circulating EDA. The same was true for complete deletion of the *Edar* gene in Ove1B mice (Headon and Overbeek 1999), indicating that endogenous EDAR does not deplete or regulate EDA levels in the circulation. EDA contains a basic domain that promotes its binding to heparin and proteoglycans (Swee et al. 2009), and it is conceivable that EDA produced in the skin, even in EDA1 transgenic mice, exerts its effects locally without systemic implication.

Regarding diagnosis of EDA-deficiency, the detection of EDA in serum, or in dried blood spots, offers an alternative to phenotypic or genetic screening of newborns. In routine dental practice, blood samples are not taken, but saliva is readily accessible. The measurement of EDA in saliva could provide useful information with regard to the cause of missing teeth. Selective tooth agenesis can be caused, among other reasons, by mutations in genes encoding master regulators of tooth formation such as PAX9 and MSX1, elements of the WNT signaling pathway such as WNT10A or AXIN2, the WNT target BMP4, or EDA, EDAR or EDARADD (Bergendal et al. 2011; Huang et al. 2013; Stockton et al. 2000; van den Boogaard et al. 2012). In cases of non-syndromic tooth agenesis, the EDA protein must be produced *in vivo* at least to some extent, however its capacity to bind to EDAR is often decreased (Mues et al. 2010). The measurement of EDA with or without pre-depletion on recombinant EDAR provides information on both expression levels and functionality of EDA.

In conclusion, this study provides the first description of soluble EDA in blood and saliva, characterizes several of its properties including receptor binding, and documents its expression

levels throughout life and disease. Although the origin and function of circulating EDA remains to be determined, its concentration and receptor-binding capacity correlate well with its function *in vivo* and could therefore be used as specific biomarkers to facilitate the diagnosis of XLHED or unexplained tooth agenesis.

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TITLES AND LEGENDS TO FIGURES

Figure 1. Human EDA levels are higher in cord blood than in the adult circulation.

A. EDA was measured in human sera, using Fc-EDA1 diluted in EDA-deficient human serum as a standard. EDA was also measured in fetal calf sera, and in sera of adult mice of the indicated genotypes. The pre-term baby at 29 weeks suffered from a generalized infection (infect). The child was 7 years old. Mean of duplicates ± SEM. Black circles: males. White circles: females.

B. Blow up of the graph with XLHED and related sera. Letters refer to sample ID of Table 1.

C. Comparison of the mean ± SEM of XLHED (HED; n=12), wild-type adult (WT; n=27), cord blood (n=12), pre-term (n=3) and fetal calf (n=3) sera. A one-way ANOVA with Bonferroni's multiple comparison tests was performed. *: $p<0.05$, ** $p<0.01$, *** $p<0.001$.

D. Blow-up of the graph with mouse sera. def: deficient. Tg: transgenic.

This experiment was performed twice with comparable results.

Figure 2. Wild-type, but not mutated EDA in serum can bind to EDAR.

A. EDA levels in normal adult sera after pre-depletion on a control protein (BCMA-Fc), or on

EDAR-Fc, or on EctoD2. Percentages of depletion with EDAR-Fc are indicated at the bottom. Mean of duplicates ± SEM.

B. Same as panel A, but for adult XLHED and related sera. Letters refer to sample ID of Table 1. Mean of duplicates ± SEM. Percentage depletion was not calculated for patient "b", because the lack of depletion on EctoD2 questioned the origin of this signal.

C. Scatter plot representation of the percentage of EDA depletion on EDAR-Fc for the indicated groups of persons.

D. AlphaLISA was used to measure Fc-EDA1 at different concentrations is assay buffer (black circles), or in saliva of an XLHED patient (opened triangles), or in serum of an XLHED patient (black inverted triangles). The lower limit of quantification (LLQ) is shown by a dotted line.

Experiments of panels A to D were performed once in the format presented. Similar results were obtained with the analysis of isolated saliva samples. Mean of duplicates ± SEM.

Figure 3. Detection of endogenous EDA in blood dried onto filter paper.

Blood from a normal adult (A), blood from an adult XLHED patient (X) and normal cord blood (C) were dried onto filter paper. 3, 10, 17, 31 and 45 days later, blood was eluted from 6 mm diameter punches of blood-impregnated filter paper, immuno-precipitated with EctoD2-coupled AlphaLISA acceptor beads and tested for the presence of EDA.

This experiment was performed once in this format. Mean of duplicates ± SEM.

Figure 4. Biochemical characterization of endogenous EDA in human and bovine sera.

A. EDA from fetal calf serum was affinity-purified on immobilized EctoD2 and EctoD3, while recombinant EDA (naturally cleaved or Flag-tagged) expressed in 293T cells were collected in

conditioned supernatant. EDA proteins were immuno-precipitated with EDAR-Fc (E) or XEDAR-Fc (X) and analyzed by Western blot with anti-EDA Renzo2 (top panels). EDAR-Fc and XEDAR-Fc were detected by re-probing the membrane with anti-Fc. Amino acid residues of EDA are indicated in brackets. PG mut: mutated proteoglycan-binding domain. GKKA, GEEA: relevant wild-type and mutated sequences in the proteoglycan-binding domain of EDA.

B. EDA proteins were (partially) digested with or without peptide N-glycanase F (PNGaseF) and analyzed by anti-EDA Western blot.

C. The indicated EDA protein were captured on heparin-coupled beads, eluted with salt, and analyzed by Western blot anti-EDA. Mut: mutated proteoglycan-binding site.

D. Gel filtration elution profiles of human sera with high (cord serum) or low (adult serum) EDA content. EDA was detected in fractions by AlphaLISA (single measures). Elution positions of molecular weight standards (in kDa) are indicated. EDA concentration in fraction of the elution of control serum was probably below the detection limit.

Experiments of panels A to D were all performed three times with similar results.

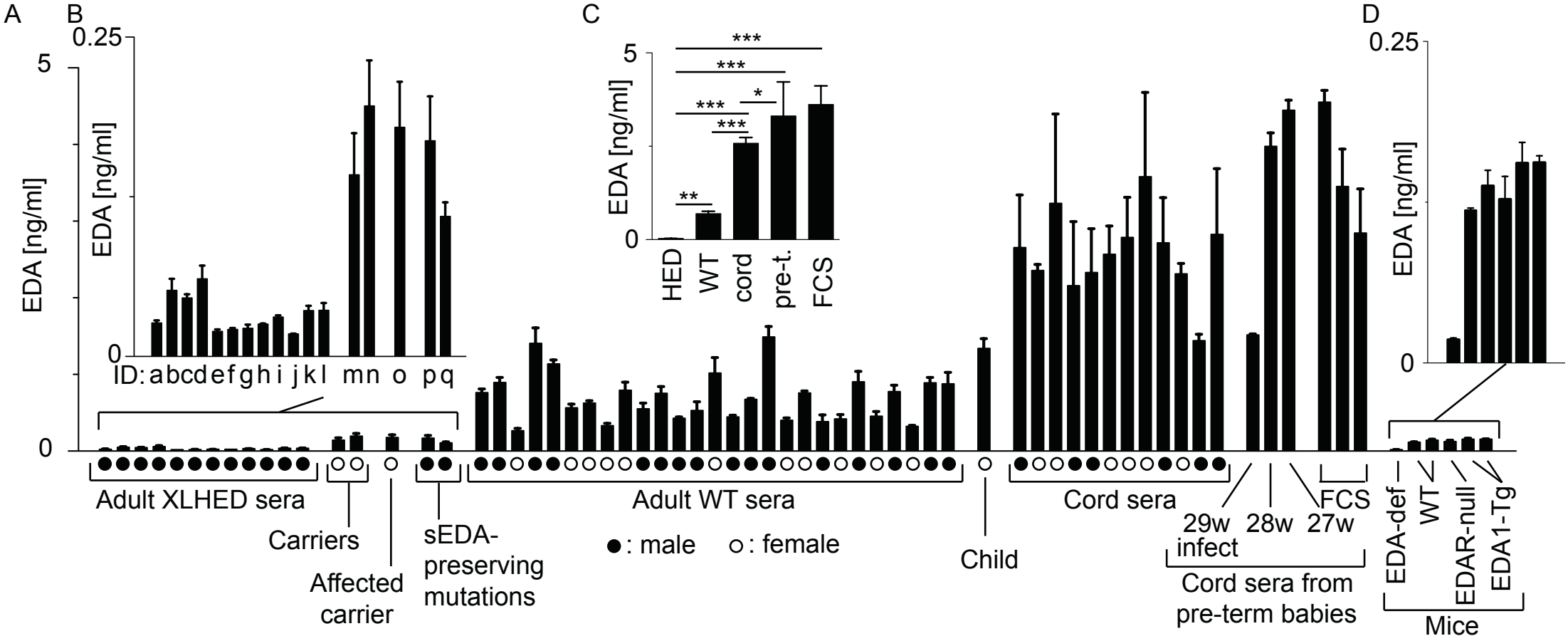
A
B
C
D
EDA [ng/ml]
5
0
0.25
ID: a b c d e f g h i j k l mn o pq
Adult XLHED sera
Carriers
Affected carrier
sEDA-preserving mutations
Adult WT sera
●: male ○: female
Child
Cord sera
29w infect
28w
27w
FCS
Cord sera from pre-term babies
HED
WT
cord
pre-t.
FCS
***
**
*
EDA-def
WT
EDAR-null
EDA1-Tg
Mice

Fig. 1

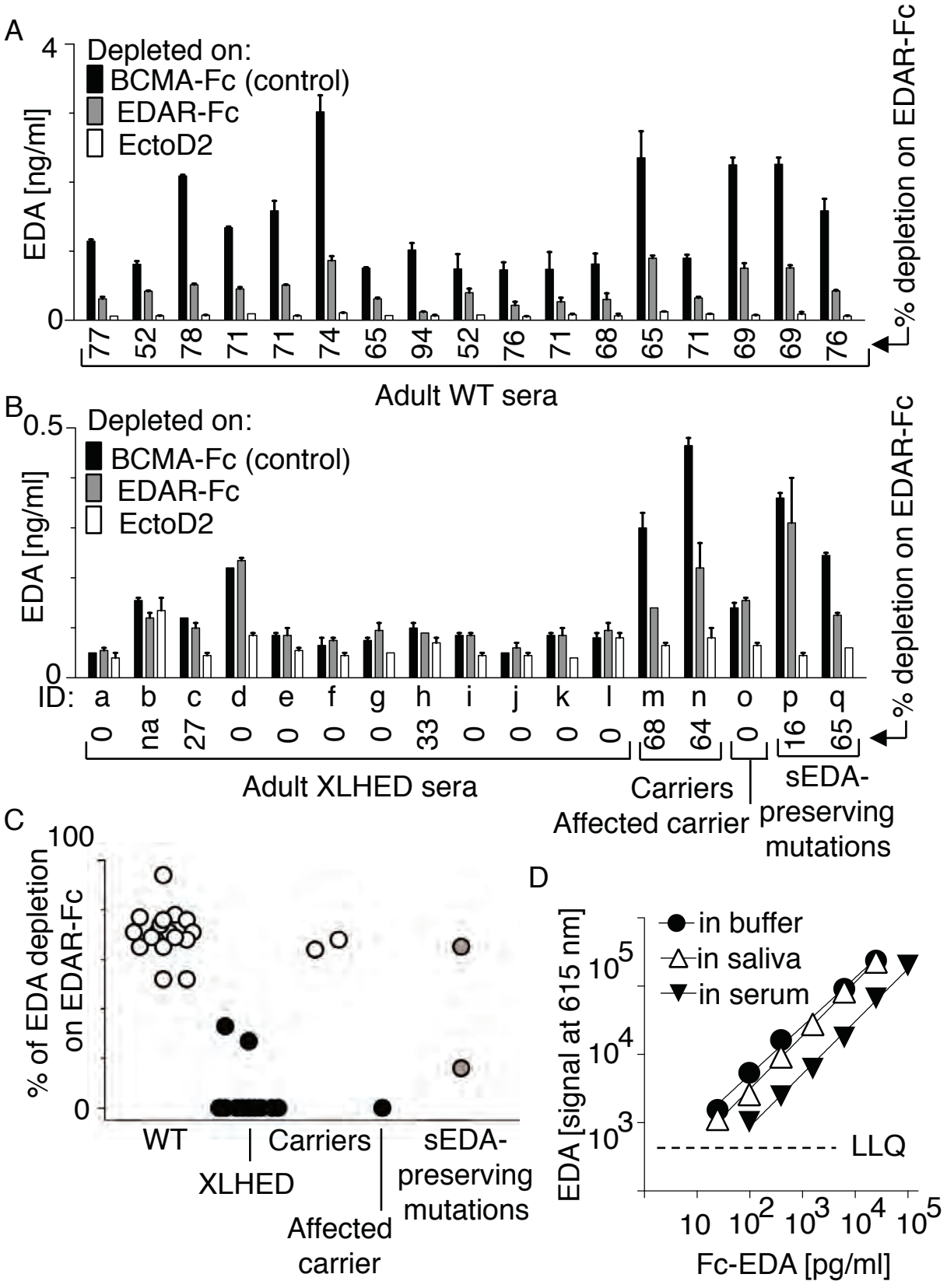
A
Depleted on:
BCMA-Fc (control)
EDAR-Fc
EctoD2
EDA [ng/ml]
4
0
% depletion on EDAR-Fc
77 52 78 71 71 74 65 94 52 76 71 68 65 71 69 69 76
Adult WT sera
B
Depleted on:
BCMA-Fc (control)
EDAR-Fc
EctoD2
EDA [ng/ml]
0.5
0
% depletion on EDAR-Fc
ID: a b c d e f g h i j k l m n o p q
0 na 27 0 0 0 0 33 0 0 0 0 68 64 0 16 65
Adult XLHED sera
Carriers
Affected carrier
sEDA-preserving mutations
C
% of EDA depletion on EDAR-Fc
100
0
WT
XLHED
Carriers
Affected carrier
sEDA-preserving mutations
D
EDA [signal at 615 nm]
in buffer
in saliva
in serum
LLQ
Fc-EDA [pg/ml]

Fig. 2

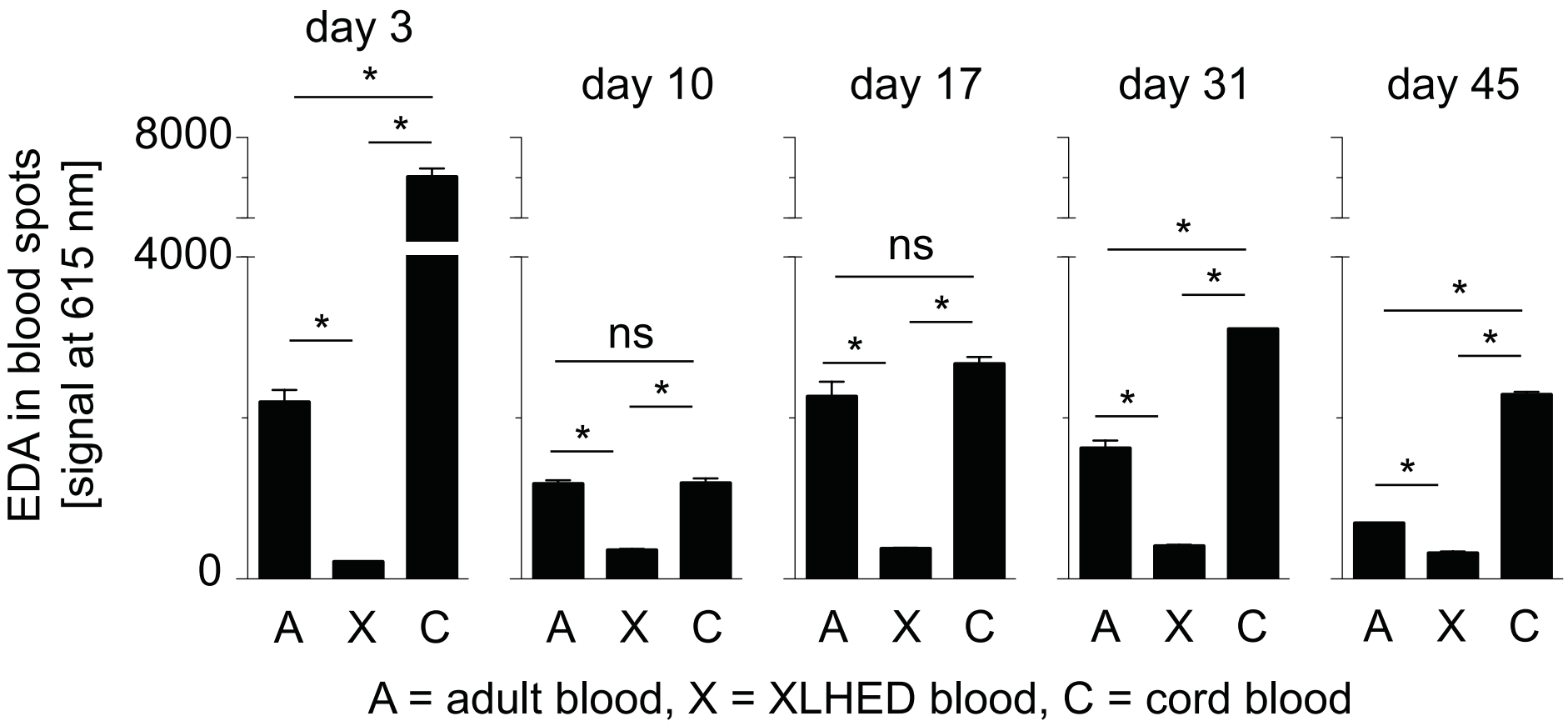
day 3
day 10
day 17
day 31
day 45
EDA in blood spots
[signal at 615 nm]
8000
4000
0
*
ns
A X C
A = adult blood, X = XLHED blood, C = cord blood

Fig. 3

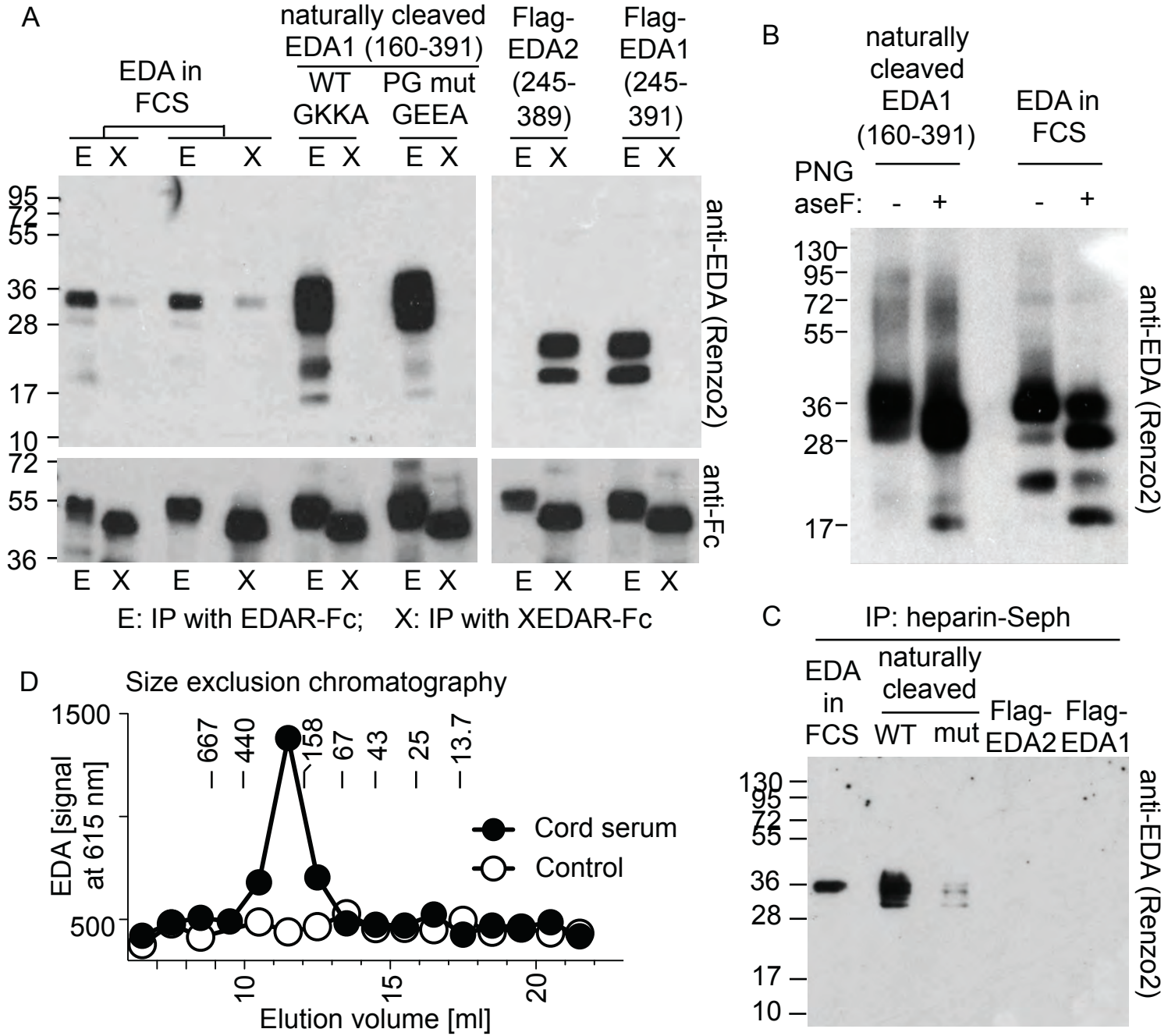
A
EDA in FCS
naturally cleaved EDA1 (160-391)
WT GKKA
PG mut GEEA
Flag-EDA2 (245-389)
Flag-EDA1 (245-391)
E X E X E X E X E X E X
95
72
55
36
28
17
10
anti-EDA (Renzo2)
anti-Fc
E: IP with EDAR-Fc; X: IP with XEDAR-Fc
B
naturally cleaved EDA1 (160-391)
EDA in FCS
PNGaseF: - + - +
130
95
72
55
36
28
17
anti-EDA (Renzo2)
C
IP: heparin-Seph
EDA in FCS
naturally cleaved
WT mut
Flag-EDA2
Flag-EDA1
anti-EDA (Renzo2)
D
Size exclusion chromatography
EDA [signal at 615 nm]
1500
500
667
440
158
67
43
25
13.7
Cord serum
Control
10
15
20
Elution volume [ml]

Fig. 4

Online supplemental appendix

Ectodysplasin A in biological fluids and diagnosis of ectodermal dysplasia.

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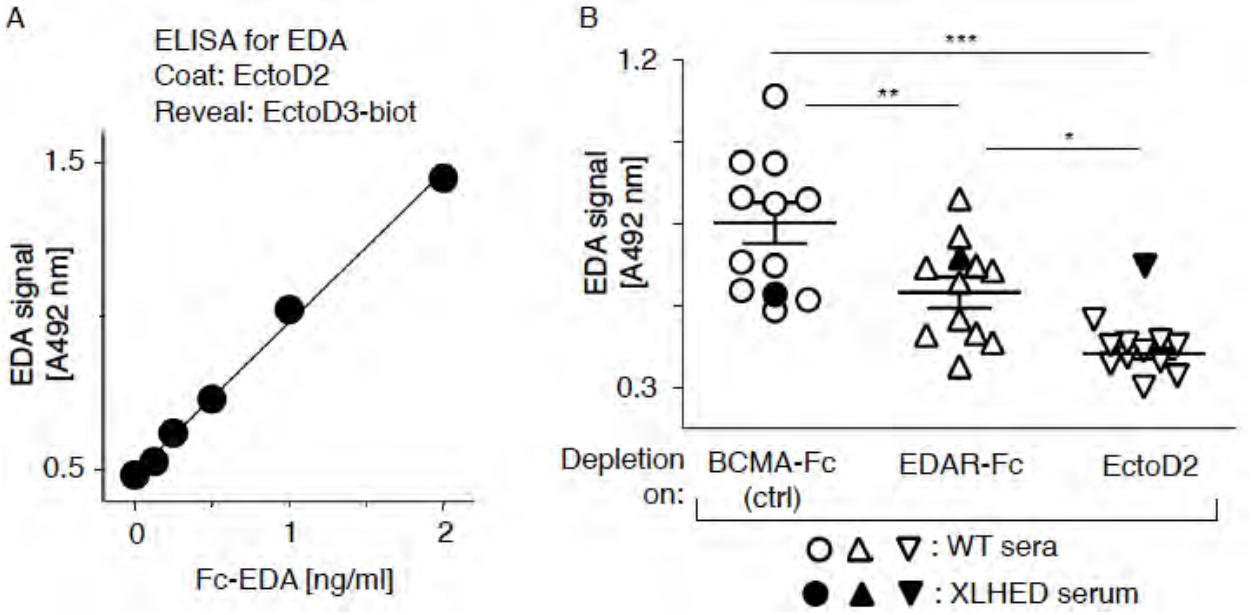
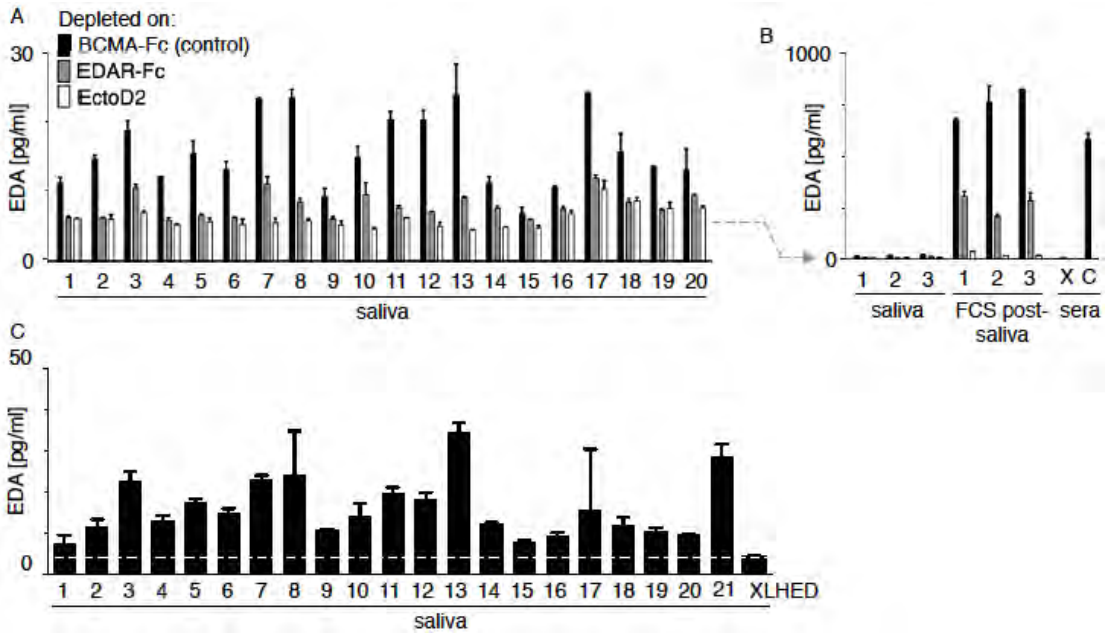


Appendix Figure 1. **Detection of EDA in human serum by ELISA**.
A. Dose-dependent detection of recombinant Fc-EDA1 in assay buffer by sandwich ELISA with a pair of anti-EDA monoclonal antibodies: EctoD2 at capture, and biotinylated-EctoD3 to reveal. B. Signal obtained with the EDA sandwich ELISA on 11 normal adult human sera (opened symbols) or one serum of an adult XLHED patient with a premature stop codon in EDA (c.64_71dupAGCCAGGG, p.C25AfsX35) (black symbols). Prior to performing the ELISA, sera were depleted twice on an irrelevant receptor (BCMA-Fc), or on EDAR-Fc, or on the capture antibody EctoD2. Result of a one way ANOVA considering WT sera only, with Bonferroni's multiple comparison test, are shown: *: $p<0.05$, ** $p<0.01$, *** $p<0.001$. The experiment was performed once in this format (and twice on isolated samples to set up conditions).

A promising sandwich ELISA for the detection of endogenous EDA in serum – A previously characterized pair of mouse IgG1 anti-EDA antibodies (EctoD2 and EctoD3) (Kowalczyk-Quintas et al. 2014) detected recombinant Fc-EDA1 with a sensitivity of about 0.3 ng/ml in a sandwich ELISA (appendix Fig. 1A); it was therefore tested for its potential ability to detect endogenous levels of circulating EDA in adult human sera. Human sera produced signals that were significantly diminished by pre-incubation on EDAR-Fc, a recombinant form of the EDA1 receptor, and even more decreased by pre-incubation with the capture antibody EctoD2 (Fig. 1B). Unexpectedly, a signal was also obtained with the serum of a patient null for the portion of the EDA protein recognized by both capture and revelation antibodies (mutation c.64_71dup AGCCAGGG,

p.C25AfsX35) (Fig. 1B). However, this signal was characterized as unspecific because it was insensitive to depletion with EDAR-Fc or with the capture antibody (appendix Fig.1B). Taken together, these results suggest that the sandwich ELISA with EctoD2 and EctoD3 anti-EDA antibodies is sensitive enough to detect endogenous levels of circulating EDA, that at least a fraction of this EDA is the EDA1 splice variant capable of binding to EDAR, but that a background signal independent of EctoD2 binding can sometimes be present and confound interpretation of results. A relatively reagent- and time-consuming strategy involving pre-depletions on the capture antibody, on EDAR-Fc and on an irrelevant receptor-Fc (BCMA-Fc) allows distinction between specific and unspecific signals. Therefore, although EDA is well-known for its involvement in embryogenesis, a circulating form can be detected in adult human sera.

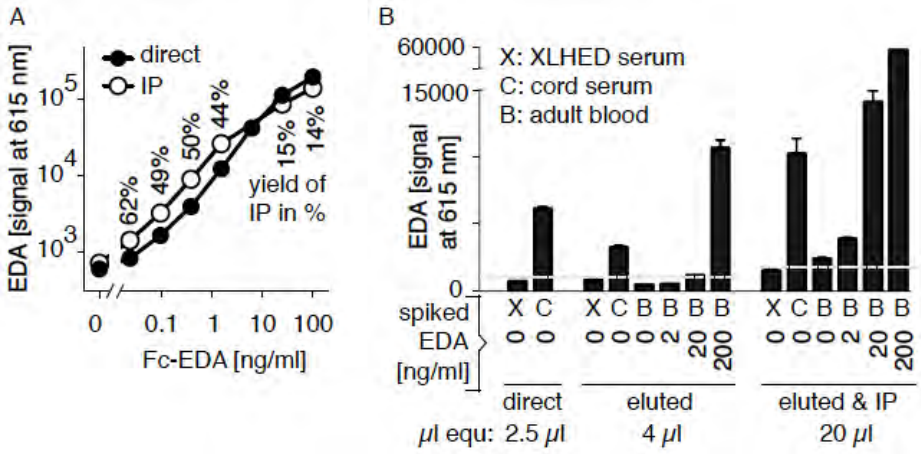
Principle of AlphaLISA – In an AlphaLISA assay, excitation of a phthalocyanine photosensitizer at 680 nm produces short-lived (4 µs) oxygen singlet, which, when reaching an acceptor bead present within its 200 nm diffusion diameter, converts a thioxene group into a di-keto derivative with concomitant light emission at 340 nm. This light is quenched by a nearby Europium chelate that emits the final signal at 615 nm (Eglen et al. 2008). This system is particularly suited for measures in sera, even hemolysed, because absorption spectra of hemoglobin and other serum proteins are low above 600 nm.



Appendix Figure 2. **Low levels of EDA can be detected in saliva.**
A. EDA levels were measured by AlphaLISA in freshly collected saliva from twenty healthy adults, after pre-depletion of the saliva on BCMA-Fc, EDAR-Fc or on the anti-EDA antibody EctoD2. EDA was quantified relative to a standard curve of Fc-EDA1 diluted in assay buffer.
B. BCMA-Fc, EDAR-Fc and EctoD2 coated in an ELISA plate were used to deplete saliva from three adults, and the exact same receptors were used immediately afterwards to deplete EDA in fetal calf serum (FCS). EDA was subsequently monitored by AlphaLISA in these samples. Sera from an XHLED patient (X) and from cord blood (C) were measured in parallel. Note that data for the saliva of volunteers 1, 2 and 3 are identical to those shown in panel A, except for the Y-axis of the graph.

C. EDA levels were monitored by AlphaLISA in frozen saliva samples from 21 healthy wild type volunteers (number 1 to 20 are identical to those of panel A, except for the freeze and thaw cycle). EDA was quantified relative to a standard curve of Fc-EDA1 diluted in saliva of an XLHED patient. The lower limit of quantification is indicated by a dotted line.
Experiments of panels A, B and C were performed once in the format presented. Similar results were obtained with the analysis of isolated saliva samples. Mean of duplicates ± SEM.

Low levels of EDA are present in saliva – An average of 15 pg/ml of EDA was detected in freshly collected saliva of twenty healthy volunteers. About 80% of this EDA could be depleted on EDAR-Fc (appendix Fig. 2A). The depletion reagents were not destroyed by saliva because they remained active to subsequently deplete EDA in a sample of fetal calf serum with high EDA levels (appendix Fig. 2B). Comparable EDA levels were found after saliva had been frozen (at -70°C), in all cases at levels higher than background measured in a saliva sample from an XLHED patient (appendix Fig. 2C). Interestingly, signals obtained with recombinant EDA in saliva of an XLHED patient versus assay buffer were only slightly reduced, indicating no major quenching effect of saliva in this assay. This contrasts with the measurement of recombinant EDA spiked into the serum of an XLHED patient, for which signals obtained were lowered 4- to 5-fold (appendix Fig. 2D). When EDA is measured in serum, it therefore appears important that the standard curve is also measured in (EDA-deficient) serum to avoid a 5-fold underestimation of serum concentrations, as seen for fetal calf serum in Appendix Fig. 2B versus Fig. 1A. In summary, low levels of active EDA are present in normal human saliva.



Appendix Figure 3. **Setup experiments for the measure of EDA in dry blood spots.**
A. 0.1 µl of EctoD2-coupled AlphaLISA acceptor beads were used to monitor Fc-EDA1 at the indicated concentrations in 5 µl of assay buffer (closed circles, “direct”). In parallel, 0.1 µl of acceptor beads were used to immuno-precipitate a five times larger volume (25 µl) of Fc-EDA1. Beads were washed and measured as performed for the direct assay (opened circles, “IP”). The calculated yields of immuno-precipitation at different concentrations of Fc-EDA1 are indicated in the graph.
B. EDA was measured directly in 2.5 µl of serum of an XLHED patient (X) or in cord serum (C). EDA was also measured in 4 µl of the same serum that had been dried onto filter paper, then

eluted in PBS, or in 4 µl of adult blood (B) that had been spiked with the indicated concentration of Fc-EDA1, then dried onto filter paper and eluted in PBS. Finally, EDA was measured in 20 µl of these same samples dried onto filter paper, eluted in PBS and immuno-precipitated with EctoD2-coupled AlphaLISA acceptor beads.

Detection of EDA in blood spots dried onto filter paper – The acceptor beads of the AlphaLISA are covalently coupled to an anti-EDA antibody and can be used to immuno-precipitate EDA with a yield of about 50% at non-saturating concentrations (appendix Fig. 3A). In addition, about a third of the content of EDA in cord blood serum could still be detected when cord blood serum was first dried overnight at room temperature on a filter paper and then eluted from the filter (Fig 5B). Upon combination of both procedures, *i.e.* when cord blood serum dried onto filter paper was eluted and then immuno-precipitated with acceptor bead, the yield of measurable EDA recovery was about 20% (appendix Fig. 3B). We then evaluated whether a similar procedure could be applied to full blood samples. When normal adult blood was dried onto filter paper, eluted and measured for EDA, values lower than those measured in XLHED serum were obtained, indicating a quenching effect of blood (appendix Fig. 3B). Indeed, eluted blood samples are rich in hemoglobin and very dark in color. Despite this, a positive signal could be obtained when blood was spiked with 200 ng/ml of EDA prior to spotting onto filter paper (appendix Fig. 3B). Most if not all of this quenching problem was overcome by EDA immuno-precipitation from eluted blood: EDA signals in normal blood were higher than those in XLHED serum and increased in a dose-dependent manner in blood spiked with increasing amounts of Fc-EDA1 (appendix Fig. 3B).

Supplemental methods

Sandwich ELISA – ELISA plates were coated with EctoD2 at 1 µg/ml in PBS, then blocked and incubated for 1 h at room temperature with 100 µl of serum that had been pre-depleted on BCMA-Fc, EDAR-Fc or EctoD2. After washing, EDA was revealed for 1 h at 37°C with 1 µg/ml of biotinylated EctoD3, followed by horseradish peroxydase-coupled streptavidin and revelation with *ortho*-phenylenediamine as described (Schneider et al. 2014). For the pre-depletions, ELISA plates were coated with BCMA-Fc, EDAR-Fc or EctoD3 at 1 µg/ml in PBS, in duplicates, then blocked and washed. 100 µl serum was applied in the first well for 6 h at room temperature, then passed to the second one overnight at 4°C, prior to transfer to the main assay plate.

AlphaLISA – Monoclonal antibody EctoD2 (50 µg) was coupled for 24 h at 37°C to 500 µg of AlphaLISA acceptor beads (Perkin-Elmer) in 200 µl of 27 mM Na-phosphate pH8, 5 mM $NaBH_3CN$ and 0.03% Tween-20. Reaction was terminated by addition of 50 µl of carboxymethoxylamine at 65 mg/ml in 0.8 M NaOH for 1 h at 37°C. Beads were washed twice in 0.1 M Tris-HCl pH8, then stored at 4°C and at a concentration of 5 mg/ml in PBS 0.05% Proclin-300. Assays were performed in white 384-well plates by mixing 5 µl of samples, usually serum diluted 1/2 in assay buffer (Perkin-Elmer), or undiluted saliva, with 20 µl of biotinylated EctoD3 at 15 ng/ml and 0.5 µg of EctoD2 acceptor beads in assay buffer. After 1 h incubation at room temperature, 1 µg of streptavidin-coupled donor beads in 25 µl of assay buffer was added. Emission at 615 nm after excitation at 680 nm was recorded with an Enspire plate reader (Perkin-Elmer). Standard curves were performed with 5 µl of recombinant Fc-EDA1 diluted into buffer, or into XLHED serum diluted 1/2, or into

XLHED saliva as indicated in the figure legends. In some cases, sera or saliva samples were pre-depleted on ELISA plates coated with BCMA-Fc, EDAR-Fc or EctoD2 as described under "Sandwich ELISA".

In some instances, recombinant Fc-EDA1, or serum, or blood, or blood spiked with Fc-EDA1 were spotted onto filter paper cards for blood sampling. After drying and storage at 4°C until use, 6 mm diameter circles were punched in the filter, placed into microtubes with a hole at the bottom, themselves placed into intact microtubes. Filters were eluted with 40 µl of PBS by centrifugation. Eluates were either measured directly by AlphaLISA, or immunoprecipitated with 0.2 µl of EctoD2-coupled acceptor beads for 1 h at room temperature. Beads were then pelleted by centrifugation at 8000 rpm (6200 x *g*) in a tabletop centrifuge, washed with assay buffer, and measured by AlphaLISA.

References for data in the supplemental appendix

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Schneider P, Willen L, Smulski CR. 2014. Tools and techniques to study ligand-receptor interactions and receptor activation by tnf superfamily members. Methods in enzymology. 545:103-125.

Appendix Table 1. **Plasmids used in this study.**

Plasmid	Designation	Protein encoded	Vector
ps508	Flag-mEDA2	HA signal-Flag-GPGQVQLQVD-mEDA1 (aa 245-389)	PCR3
ps548	Flag-mEDA1	HA signal-Flag-GPGQVQLQVD-mEDA1 (aa 245-391)	PCR3
ps739	hBCMA-Fc	Ig signal-EVKLVPRGS-hBCMA (aa 2-54)-VD-hIgG1 (aa 245-470)	PCR3
ps930	hEDAR-Fc	hEDAR (1-183)-VD-hIgG1 (aa 245-470)	PCR3
ps1060	hXEDAR-Fc	HA signal-DVT-hXEDAR (aa 1-133)-VD-hIgG1 (aa 245-470)	PCR3
ps1752	hEDA1 full	hEDA1 (aa 1-391)	PCR3
ps1938	Fc-EDA1	HA signal-hIgG1 (aa 245-470)-hEDA1 (aa 238-391)	PCR3
ps2867	hEDA1 full HBD mut	hEDA1 (aa 1-391) KK177/178EE	PCR3

Flag = DYKDDDDK HA signal=MAIIYLILLFTAVRG
Ig signal=MNFGFSLIFLVLVLKG HBD mut : heparin binding domain mutant