

Accepted Manuscript

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PII: S0091-6749(17)31878-X

DOI: [10.1016/j.jaci.2017.10.036](https://doi.org/10.1016/j.jaci.2017.10.036)

Reference: YMAI 13144

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 30 May 2017

Revised Date: 28 September 2017

Accepted Date: 23 October 2017

Please cite this article as: Bonura A, Di Blasi D, Barletta B, Butteroni C, Corinti S, Gervasi F, MelisMario R, Uasuf C, Fabio C, Di Felice G, Colombo P, Modulating allergic response by engineering the major *Parietaria* allergens, *Journal of Allergy and Clinical Immunology* (2018), doi: 10.1016/j.jaci.2017.10.036.

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Modulating allergic response by engineering the major *Parietaria* allergens

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Keywords: *Parietaria judaica*; Allergen Specific Immunotherapy; hypoallergenic hybrid; PBMC; mouse

Capsule Summary: An engineered recombinant hybrid composed of the two major *Parietaria* allergens (PjEDcys) with reduced allergenicity, retained immunogenicity but immunomodulatory activity for Allergen Immunotherapy of *Parietaria* allergic patients.

To the Editor

Allergen Immunotherapy (AIT) is a curative approach capable of targeting the immunological causes of allergic reactions, ameliorating symptoms and stopping the progression of the disease. Clinical studies have demonstrated that long-term clinical improvement is associated with humoral and cellular modifications, such as a decrease in effector cells in target organs, a marked increase in IgG antibodies and in regulatory T (Treg) and B cells¹. In recent years, novel immunotherapy strategies, such as the design of standardized recombinant hypoallergenic derivatives, have been tested in clinical trials, showing that the AIT strategy of reducing allergic adverse events represents a promising therapeutic approach².

Parietaria judaica (Pj) pollen allergenic components (Par j 1 and Par j 2) have been investigated, showing that they belong to the Lipid Transfer Proteins (LTPs), one of the most relevant families of allergenic proteins in pollen and plant-derived food³. Engineered hybrids of the two major *Parietaria* allergens have been already developed to obtain hypoallergenic molecules potentially applicable to allergy immunotherapy⁴. Our research group developed strategies to design and characterize a novel engineered hybrid composed of the two major *Parietaria* allergens (PjEDcys) with reduced allergenicity but retained immunogenicity, in both murine and human models⁵. In this scenario, we decided to study the modulation of the immunological response of the disulphide bonds PjEDcys hybrid (Figure 1 panel A) in Peripheral Blood Mononuclear Cells (PBMC) from *Parietaria* allergic patients and in a mouse sensitization model.

Our previous work demonstrated that the major allergens of Pj pollen are capable of inducing both the innate and adaptive arms of the human immune system in healthy subjects⁶. Following this line of evidence, we first observed that PjEDcys can induce a proliferative response in both CD4⁺ and CD56⁺ lymphocytes in a way comparable to the wild type allergens in allergic patients (n=10), demonstrating that mutagenesis did not interfere with lymphocyte proliferation (Supplementary materials and methods; summary of the data and statistics are shown within Tables E1 and E2). Decades of research on human and animal models provide evidence that inflammatory response in allergic disease is associated with Th2 cytokines. Among them, IL-5 and IL-13 are responsible for many of the important features of airway inflammation and remodeling in patients with allergic asthma⁷. Figure 1 panels B and C shows that stimulation of PBMC from Pj allergic patients (n=10) with PjEDcys induces a statistically significant reduction in the percentage of CD4⁺ cytokine secreting cells (p=0.037 and p=0.028, respectively) when compared to wild type recombinant allergens. Furthermore, following the well-established clinical evidence that successful AIT is characterized by the induction of Treg, we looked at the ability of PjEDcys to induce CD4⁺CD25⁺CD127⁻ cells in allergic patients (n=6). This approach showed that the hybrid increases

the number of this cell subset starting from day 7, reaching statistically significant induction at day 8 ($p=0.043$) (Figure 1 panel D) (Supplementary materials and methods for details).

We performed one preliminary mouse study of *Parietaria* sensitization to provide proof of concept for the *in vivo* therapeutic potential of PjEDcys. The hybrid was administered subcutaneously (S.C.) in two different doses (10, 50 $\mu\text{g}/\text{mouse}$) every other day for three total exposures in mice previously sensitized with the Par j 1/Par j 2 Mix ($n=6$ mice/group). A third group ($n=6$ mice) was treated with PBS only as control (Figure 2, panel A). At the end of the treatment, specific antibody responses to the Par j 1/Par j 2 Mix were evaluated (Figure 2, panel B). Serum was collected and Par j 1/Par j 2 Mix-specific IgE, IgG1, IgG2a levels were measured by ELISA. A trend towards a dose-response effect of the treatment was observed for the three isotypes. The results indicate that therapeutic treatment with the higher dose of PjEDcys (50 $\mu\text{g}/\text{mouse}$) was able to significantly reduce the levels of specific IgE in comparison with the PBS-treated group ($p < 0.005$). Conversely, IgG1 and IgG2a production was significantly increased by the therapeutic treatment at the same dose ($p < 0.01$ for both isotypes) (Supplementary materials and methods for details). It is well known that blocking IgG antibodies, mainly belonging to human IgG4 and IgG1 isotypes, are formed during the course of successful AIT in allergic patients and are induced by high doses of antigen⁸. In our mouse study, both IgG1 and IgG2a isotype levels were increased by PjEDcys therapeutic treatment, corresponding to human IgG4 and IgG1 isotypes, respectively. Moreover, the *in vivo* therapeutic study demonstrates the capability of the engineered hybrid to modulate the pre-existing sensitization towards a protective humoral response.

The modification of the structure of an allergen to increase vaccine safety has previously been suggested by several authors² but, our results additionally support the hypothesis that the modification of the protein structure of an allergen can modulate the immune response, reducing the induction of Th2 cytokine secreting cells towards a more tolerogenic immune response. Furthermore, the therapeutic mouse model of sensitization to *Parietaria* allergens reinforced our findings, suggesting that it is possible to design an engineered recombinant allergen hybrid with improved safety (hypoallergenicity), retained immunogenicity, and the ability to modulate immune response. These observations find support from data in the literature where it has been proposed that, for every given protein, an optimal conformational stability can be relevant for the efficient processing and presentation of T cell epitopes; this demonstrates that different ranges of stability may polarize an immune response towards a Th1, Th2 or Treg response, enhancing or inhibiting antibody response⁹. Finally, it is noteworthy that we propose a strategy based on the disruption of disulphide bonds, which is a signature for all allergens belonging to the LTP family (ubiquitous allergens that are found in divergent plant species sharing structural features), suggesting that our

strategy could open the way to a powerful approach for a large set of molecules belonging to this allergen family.

Acknowledgments

Biomay AG Vienna funded this work

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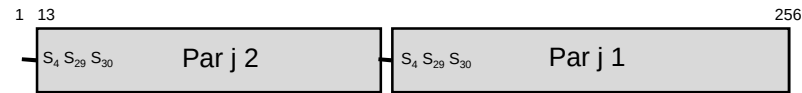
Figure Legend

Figure 1.

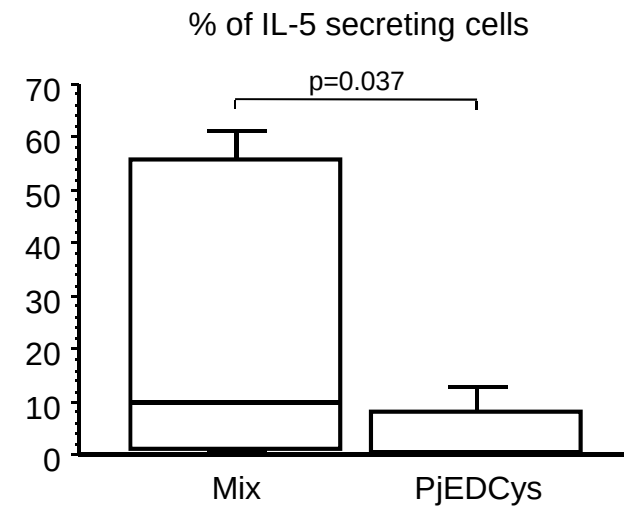
Schematic representation of the PjEDcys hybrid structure (panel A). Grey boxes indicate full-length allergenic sequences. Letters and numbers within the boxes indicate the position and cysteine to serine substitutions. Numbers on top of the panel indicate hybrid size. Changes in the percentage of CD4⁺ cytokine secreting cells (panels B and C) and CD4⁺CD25⁺CD127⁻ cells in allergic patients (panel D) after stimulation with PjEDcys compared to the Mix (Par j 1/ Par j 2). For each box plot (panels B-D), horizontal bars indicate (from the bottom to the top) 10th, 25th, 50th (median), 75th, and 90th percentiles. Values below 10th and above 90th percentiles are plotted as circles. P values were computed by Mann-Whitney U-test (panels A-B) and Kruskal-Wallis test (panel C).

Figure 2. Panel A) Experimental design of *in vivo* sensitization and therapeutic treatment. Panel B) Therapeutic treatment with PjEDcys decreases serum levels of rPar j 1+rPar j 2-specific IgE and increases rPar j 1+rPar j 2-specific IgG1 and IgG2a. Specific antibodies were evaluated in serum samples collected after therapeutic treatment (day 42) from groups of mice treated with two doses of PjEDcys or PBS as control.

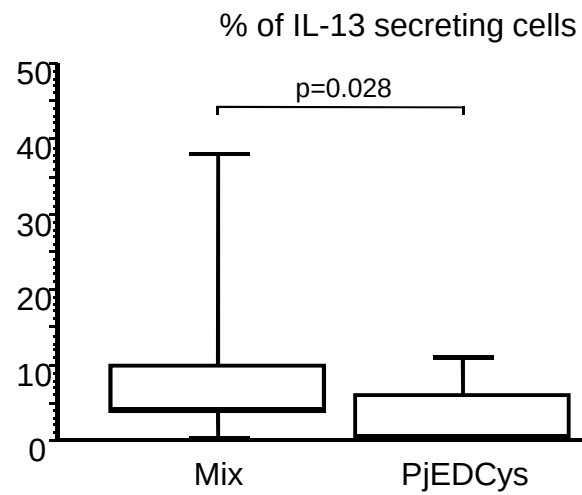
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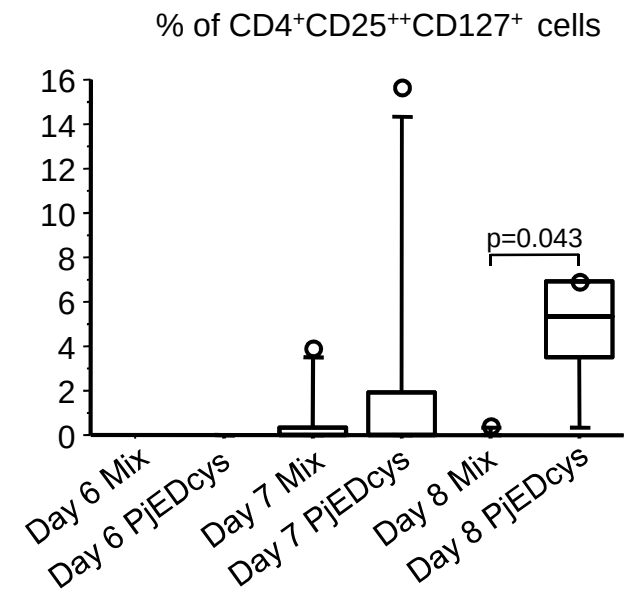
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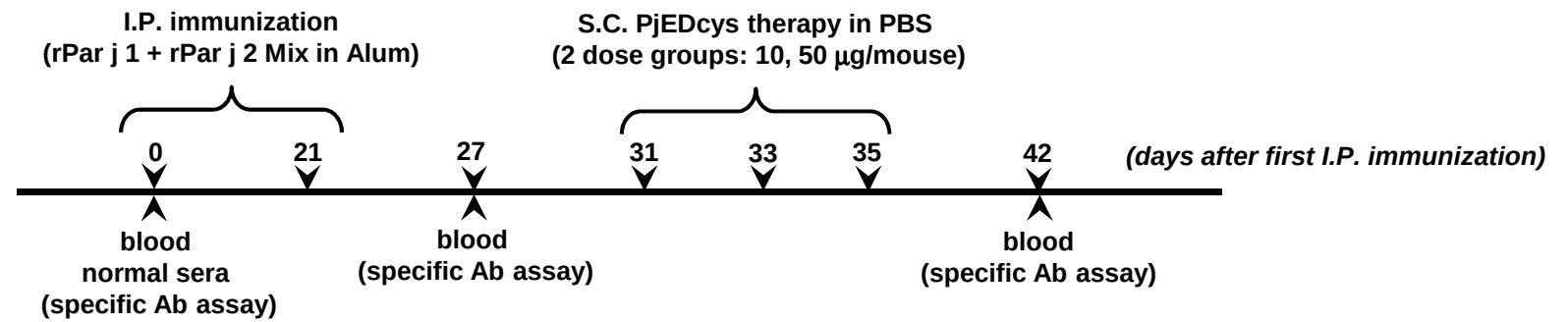
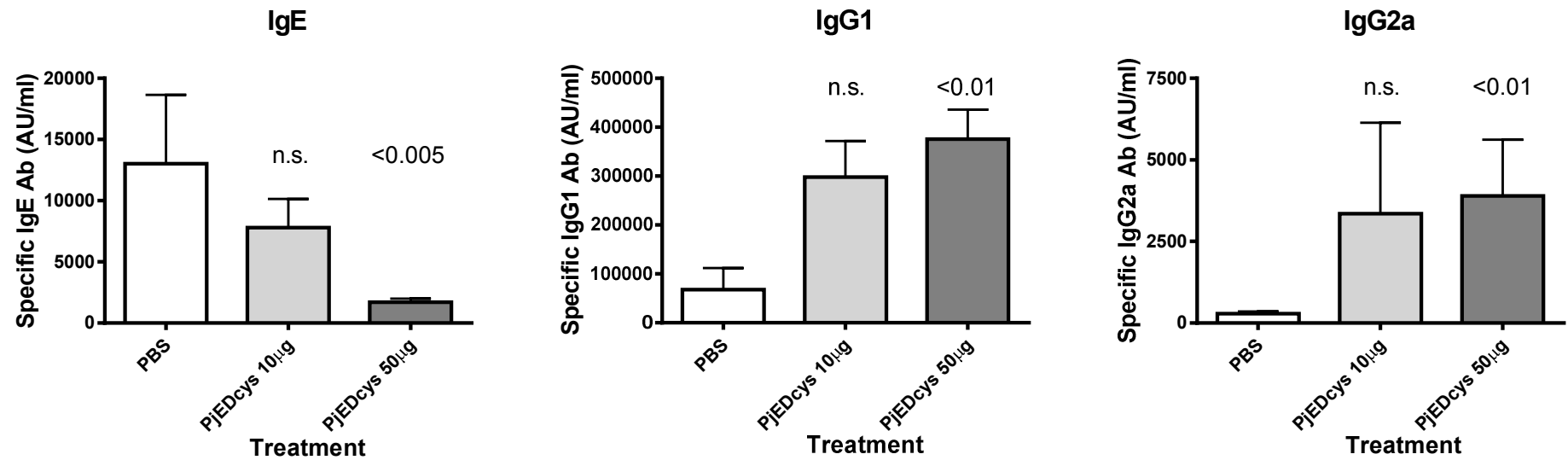


C



D



A**B**

Recombinant allergens

Recombinant allergens were expressed as His-tagged proteins (pQE30 vector, QIAGEN, UK) in the M15 strain (QIAGEN, UK). Induction and purification protocols were conducted as previously described¹.

Recombinant proteins were tested for the endotoxin content using the Multi-test Limulus Amebocyte Lysate (LAL) pyrogen plus test (Bio-Whittaker, USA) (endogenous endotoxin content ≤ 0.003 ng LPS/ μ g of recombinant protein). The allergens used in this study were the rPar j 1, rPar j 2 and the rPjEDcys.

CFSE proliferation assay

Peripheral Blood Mononuclear Cell (PBMC) from 10 Pj allergic patients were prepared from blood from *Parietaria* allergic donors by standard Ficoll density gradient separation and resuspended in 1XPBS pH 7.2 (1×10^7 cells/ml) and labelled with a solution containing 5 μ M Carboxy-Fluorescein Diacetate Succinimidyl Ester (CFDA-SE) (Molecular Probe) as previously described¹. Cells were incubated with the antigens (rPar j 1 plus rPar j 2 [mix] and rPjEDcys) at a concentration of 1 and 10 μ g/ml for 7 days. LPS (1 μ g/ml) was used as a positive control. Then CFSE-labelled cells were stained with an anti-CD4 PE and anti-CD56 PercPCy5.5 antibodies for 20 min at 4°C in the dark. Background proliferation was measured in the unstimulated sample (medium alone). A minimum of 50,000 events were collected and analysed on a FACScalibur flow cytometer. Analysis gates were set on live lymphocyte by forward and side scatter profile. Results were analysed using the WinMDI 2.8 software (see Table E1 and E2)

Cytokines secretion assay

PBMC was prepared from blood from *Parietaria* allergic donors by standard Ficoll density gradient separation. This study has been approved by the Institutional ethical committee and donors signed an informed consent before undergoing the study protocol.

Cells were resuspended at a concentration of 5×10^6 /ml in RPMI 1640 complete medium with 25 mM HEPES, L-Glutamine and supplemented with Penicillin (100U/ml) and Streptomycin sulfate (100 mg/ml) (Invitrogen), 10% Human AB serum (Euroclone, Milan, Italy), 1% Non-Essential Amino Acid (AAANE) (Euroclone, Milan, Italy) and 1 mM Sodium Pyruvate (Euroclone, Milan, Italy). PBMC were seeded in 24-well tissue cultures plate stimulated with 20 μ g/ml of the recombinant allergens Par j 1/Par j 2 mix and PjEDcys for 14 hours. Stimulation of all samples, including unstimulated cells, was done in the presence of (10 μ g/ml) anti-CD28 and anti-CD49d

monoclonal antibody cocktail (BD Biosciences-USA). Cytokine secretion assays were performed according to the manufacturer's instructions (Miltenyi Biotech). All cytokines studied were stained with anti-PE detection antibodies; the cells were collected and stained with the following mAbs: anti-CD14 PerCP (Miltenyi Biotech), anti-CD4 FITC (Miltenyi Biotech) for 20 min at 4°C in the dark. Immediately before FACS analysis, Propidium Iodide (PI) (0.5 mg/ml, Sigma) was added to each sample to distinguish live and dead cells. A lymphocyte gate based on forward and side scatter properties was set. Dead cells and monocytes were excluded according to PI and CD14-PerCP staining on a FL2 versus FL3 plot. A minimum of 250,000 events were collected and analyzed on a FACScalibur flow cytometer (Becton Dickinson) and data were analyzed using the Win MDI 2.8 software.

Immunophenotyping

PBMC from *Parietaria* allergic patients (n=4) were cultured in RPMI complete medium with 10% FCS serum at a concentration of 0.5×10^6 cells/ml, seeded in 24 well tissue culture plate and incubated with the rPar j 1/ rPar j 2 mix and the PjEDcys at 10 mg/ml for 8 days. Cells were harvested every day (from 1 to 8 days), washed in cold FACS Buffer (1XPBS-1%FBS-0.1% sodium azide) and stained with anti-CD4 FITC (Caltag) and anti-CD25 PE (Miltenyi Biotech) for 20 min at 4°C in the dark. A minimum of 50,000 events were collected and analyzed on a FACScalibur flow cytometer. Data were analyzed using the Win MDI 2.8 software. Analysis gates were set on live lymphocyte by forward and side scatter profile. Then, since all the four patients showed a very low increase of induction of CD4⁺/CD25⁺ from day 1 to 5, ten additional patients were studied from day 6 to 8 (data not shown).

PBMC from *Parietaria* allergic patients (n=6) were cultured in RPMI complete medium with 10% FCS serum at a concentration of 0.5×10^6 cells/ml, seeded in 24 well tissue culture plate and incubated with the rPar j 1/ rPar j 2 mix and the PjEDcys at 10 mg/ml for 8 days. Cells were harvested every day (from 6 to 8 days), washed in cold FACS Buffer (1XPBS-1%FBS-0.1% sodium azide) and stained with anti CD4-PerCp (Miltenyi), anti CD25-PE (Miltenyi Biotech) and anti CD127-FITC (Miltenyi Biotech) for 20 min at 4°C in the dark. A minimum of 50,000 events were collected and analyzed on a FACScalibur flow cytometer. Data were analyzed using the Win MDI 2.8 software. Analysis gates were set on live lymphocyte by forward and side scatter profile. Then cells were analyzed according to the expression of the CD4⁺ CD25⁺ antigens. Furthermore, within this population, the expression of the CD127 antigen was evaluated.

The data were summarized in the Figure 1 panel C

In vivo therapeutic treatment with PjEDcys

BALB/c female mice were intraperitoneally (I.P.) sensitized with an equimolar mixture containing 2 µg of rPar j 1 and 1.65 µg of rPar j 2 (Par j 1/Par j 2 MIX, 0.150 nM of each recombinant allergen) on days 0 and 21, according to a previously published protocol ². The PjEDcys was used as a therapeutic agent in two different doses (10, 50 µg/mouse) subcutaneously (S.C.) administered three-times every other day (days 31, 33, 35) in mice previously sensitized with the Parj1/Parj2 MIX (n=6 mice/group). A third group (n=6 mice) was treated with PBS only as control. The experimental protocol is reported in Figure 2, panel A. Blood samples were collected before sensitization and on days 27 (after sensitization) and 42 (after therapeutic treatment). Specific antibody responses (IgE, IgG1, IgG2a isotypes) to the Par j 1/Par j 2 MIX have been evaluated in ELISA assays as previously described ³.

Statistical Analysis

Ex Vivo assays. Differences among subgroups were evaluated by means of tests for not normally distributed data (Mann-Whitney U-test and Kruskal-Wallis test).

In vivo animal assays. Groups of 6 mice/treatment were used throughout the study. Data were presented as means with SEM. The nonparametric Mann-Whitney U-test was used to compare each experimental group with the relevant control group. P values <0.05 were considered significant.

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Percentage of CD4⁺ proliferating cells in *Parietaria judaica* allergic patients

	rPar j 1 + rPar j 2	rPjEDcys	rPar j 1 + rPar j 2	rPjEDcys
	1 µg/ml		10 µg/ml	
Patient	p=0.647		p=0.169	
1	9.1	14.5	29.1	13.4
2	23.5	21	32.2	21.7
3	23.8	35.9	31.8	18
4	0	27.4	6.6	12.2
5	0.4	0.5	0.8	0.4
6	5.7	0	6.6	0
7	23.5	24.4	25.4	29.3
8	8.6	2.9	11.7	12.8
9	1.2	0.8	4.6	3
10	2.3	2.5	4.4	4
Median and IQ range	7.15 (1.20-23.50)	8.70 (0.80-24.40)	9.15 (4.60-29.10)	12.50 (3.00-18.00)

IQ range: interquartile range

Percentage of CD56+ proliferating cells in *Parietaria judaica* allergic patients

	rPar j 1 + rPar j 2	rPjEDcys	rPar j 1 + rPar j 2	rPjEDcys
	1 µg/ml		10 µg/ml	
Patient	p=0.906		p=0.386	
1	0.1	0.1	0.3	0.6
2	0.4	0.2	1.3	1.1
3	0.6	0	1.4	0
4	0.5	0.2	1	1.1
5	0	0.3	0.2	0.3
6	0.2	0.9	0.3	1.3
7	0	0.1	0.5	0.6
8	0.1	0.4	3.2	1.5
9	0.4	0.1	1.5	0.7
10	0.1	0.3	1.1	0.4
Median and IQ range	0.15 (0.10-0.40)	0.20 (0.10-0.30)	1.05 (0.30-1.40)	0.65 (0.40-1.10)

IQ range: interquartile range