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ICTP concentration in cervical-vaginal fluid as a potential marker of membrane collagen degradation before labor

Running title: ICTP concentration in cervical-vaginal fluid before labor

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ABSTRACT

Objectives: Numerous physical and chemical processes lead to rupture of membranes. Within the fetal membranes there are numerous types of metalloproteinases, which cause collagen type I degradation. The C-terminal telopeptide of colagen type I (ICTP) is the breakdown product of type I collagen. The aim of the study was to determine whether ICTP is secreted

into the vaginal-cervical fluid (VCF) in the case of physiological rupture of the membranes of the fetus before delivery.

Material and methods: The study was conducted in March 2021 at the Department of Obstetrics and Perinatology of the Jagiellonian University in Cracow, Poland. Twenty-three cases were included in the study. During routine gynecological examination with the use of specula, VCF was collected twice in a volume of 50 μL. The obtained material was then subjected to enzyme immunoassay using the Human C-telopeptide of type I collagen (ICTP) ELISA Kit (Catalog Number. CSB-E10363h). The concentration of ICTP in the sample was calibrated. The concentration range that the device can detect was 25 ng /mL–800 ng/mL.

Results: The presence of ICTP in the VCF was confirmed. The minimum concentration was 43.72 ng/mL, the maximum was 762.59, in five cases the concentration was outside the maximum scale of the device.

Conclusions: ICTP was confirmed in the VCF of pregnant women before physiological delivery. Further studies are required to accurately evaluate ICTP as a marker of the processes of collagen degradation in fetal membranes in the mechanism of physiological labor and premature rupture of the membranes.

Key words: ICTP; pPROM; rupture of membranes; preterm delivery

INTRODUCTION

Rupture of the membranes (ROM) consists of numerous physical and chemical processes, from mechanical stretching to the metabolism of the components of the fetal membranes and intercellular matrix. Within the fetal membranes there are numerous types of metalloproteinases, of which MMP-2 and MMP-9 are the most studied, and MMP-1 and MMP3 slightly less [1, 2]. MMP-2 and MMP-9 cause collagen type I degradation of which one of the products is ICTP, the carboxyterminal telopeptide of type I collagen [3]. In the literature there is a lack of studies assessing the secretion of ICTP into vaginal-cervical fluid (VCF) in the case of physiological ROM.

Objectives

The aim of the study was to determine whether ICTP, as a marker of type I collagen degradation, is secreted into the VCF during ROM before physiological delivery. This study is the beginning of studies determining the concentration of ICTP in VCF during physiological ROM and in the event of premature ROM. The study may check the usefulness of ICTP in relation to pregnant women in predicting preterm premature ROM.

MATERIAL AND METHODS

This prospective non-randomized study was conducted in March 2021 at the Obstetrics and Perinatology Department of University Hospital in Krakow, Poland. The patients who were enrolled for the study underwent routine gynecological examination with the use of a speculum VCF from the external cervix was obtained using a pipette. Approximately 50 μL of material was collected in Eppendorf tubes. During the same gynecological examination, material was collected twice from each patient in order to eliminate concentration measurement errors. Prior to immunoenzymatic testing, the material was stored at –80°C. Subsequently, the collected samples were simultaneously assayed with the use of a kit, the human C-telopeptide of type I collagen (ICTP) ELISA Kit (Catalog Number. CSB-E10363h) by using spectrophotometry. The calibration standards were assayed at the same time as the samples. The concentration range that the kit can detect is from 25 ng/mL to 800 ng/mL.

Patients participating in the study met the following inclusion criteria:

a) gestational age between the 38th and 42nd week of pregnancy,
b) single pregnancy,
c) c) age ≥ 18 years old,
d) lack of traits characteristic of infection of the genital tract.

Exclusion criteria from the test:

a) gestational age: < 38 weeks of pregnancy or > 42 weeks of pregnancy,
b) multiple pregnancy,
c) 2nd or 3rd stage of labor,
d) rupture of membranes (detected by means of physical examination or in case of doubt by assessing the change in pH of the vagina from acidic to alkaline),
e) active infection of the genital tract.

Statistics

All calculations were performed using R software (Development Core Team, Vienna, Austria, version 4.0.4). All tests were two-sided and statistical significance was defined as $p < 0.05$. The standard curve of Optical Density versus ICTP concentration was used to determine the amount of ICTP in an unknown sample. The curve was generated by plotting the average Optical Density from two measurements obtained for each of the six standard concentrations 0, 25, 75, 175, 400 and 800 ng/mL on the vertical axis versus the corresponding concentration on the horizontal axis. We fitted nonlinear regression with the ‘nls ()’ function to estimate parameters of the curve. This nonlinear least-squares algorithm is an iterative procedure

requiring specification of starting values for the parameters. We got starting values automatically applying self-starting asymptotic regression function. The concentration of ICTP in the samples was then determined by comparing the Optical Density of the samples to the standard curve. Inverse transformation allowed to predict the confidence intervals of ICTP concentrations for each participant. When estimated concentration was outside the range that the device can detect (25 ng/mL–800 ng/mL) the result was classified as “not detectable”.

The study received bioethical commission approval. The patients who took part in the study obtained information about the study and expressed their written consent to participate in it.

RESULTS

The Obstetrics and Perinatology Clinic of Jagiellonian University in Krakow, Poland is a tertiary healthcare center; there were 201 births during the study period, including 99 via cesarean section. 40 cases did not meet the inclusion criteria (mainly due to rupture of membranes or delivery before 38 weeks of pregnancy). The remaining patients did not agree to participate in the study. In total, 41 patients were enrolled, however in 18 patients the researchers didn’t manage to take the samples. A flow chart of the recruitment of women giving births is shown in Figure 1.

Characteristics of the study group

The median age of the study group was 31 years, the gestational age was 39.29 weeks, and the BMI before pregnancy was 21.61 kg/m^2. In the study group, 33.3% of patients gave birth for the first time. The data are presented in Table 1.

According to the guidance of Authors of the Human C-telopeptide of type I collagen (ICTP) ELISA Kit, we firstly constructed own standard curve of Optical Density versus ICTP concentration. The necessity of such approach was caused by a high sensitivity in result to any variation in reagent preparation and assay procedure (e.g., pipetting, washing technique, incubation time, temperature, kit age). Each of six standard ICTP concentrations has been assigned to associated optical density (see Fig. 2). Next, we used to obtain standard curve as the reference to determine the concentration of ICTP in our sample.

The results are shown in Table 2. Both optical density measurements I and II were similar, which indicates the test–retest reliability. The final calculated values are the average concentration of ICTP obtained from two samples. In five cases, the concentration of ICTP was so high that it exceeded the upper limit of the kit in both measurements. However, in one

case it was below the lower limit. In 10 patients there the researchers didn't manage to take the second sample.

In further research, hierarchical cluster analysis was performed, and two clusters were separated based on the mean ICTP concentration. Then, patients from the study group were compared in the high concentration subgroups - group 1 (median ICTP 2.01 ng/mL) and the lower concentration — group 2 (median ICTP 0.44 ng/mL). The patients did not differ in terms of age, duration of pregnancy, BMI, and fertility. It was noticed that in group 2 patients suffered from hypothyroidism more often. Moreover, in gynecological examination, in group 1 shortening of the cervix was observed more frequently ($p = 0.033$). No difference in ICTP concentration was observed when analyzing dilation and Bishop scale. The results are presented in Table 1.

DISCUSSION

Although pregnancy and childbirth have accompanied humans since the dawn of time, compared to other fields of medicine, knowledge about the molecular mechanisms occurring during pregnancy and childbirth is small. Admittedly, significant progress has been made in the field of obstetrics, perinatology and neonatology in recent years, but it is mainly based on the improvement of symptomatic treatment methods, and not on interventions aimed at the pathogenesis of the pathological process. In recent decades, perinatal mortality has significantly decreased, but so far it has not been possible to explain the reasons leading to the occurrence of such diseases as premature rupture of membranes leading to preterm delivery [4–6].

Preterm birth, defined as delivery before 37 weeks of gestation, still is a large percentage of neonatal mortality and morbidity [7–9]. Premature rupture of membranes (PROM) is when the amniotic sac ruptures before contraction. Depending on the time of rupture of membranes, before vs after 37 weeks of pregnancy, it is divided into the preterm premature rupture of membranes (pPROM) and term PROM. This complication occurs in 3% of pregnancies and is considered the strongest risk factor for preterm delivery [4, 10]. Currently, the recommended treatment is empiric antibiotic therapy, especially when the state of vaginal culture for group B streptococcal carriers is unknown [11–14]. An innovative procedure is the combination of antibiotic therapy with intravaginal probiotic treatment [15]. According to the recommendations, the supply of steroids is recommended [16]. In case of signs of intrauterine infection or placental abruption, the end of pregnancy is necessary. In the absence of such symptoms, delivery after 37 weeks is recommended [17, 18]. Studies show

that expectant management in twin pregnancies is associated with a similar prognosis for newborns as in the case of singleton pregnancies [19].

Neonatal complications are mainly due to prematurity. However, oligohydramnios and chorioamnionitis also play an important role. The occurrence of these complications is associated with neonatal sepsis, neurodevelopmental disorders, and bronchopulmonary dysplasia. The postpartum outcome is difficult to predict during pregnancy. This is associated with enormous stress for the mother, the lack of optimal prenatal consultation and the possibility of individualized treatment [20–24].

In the literature, various biomarkers/proteins associated with the onset of preterm delivery are checked. A known and commercially tested protein is placental alpha microglobulin-1 (PAMG-1). Sensitivity (SN), specificity (SP), positive predictive value (PPV) and negative predictive value (NPV) of prediction of PTB within seven days were 50%, 80.56%, 12.5% and 96.67%, respectively [25]. Another promising test was the assessment of progesterone gene polymorphism. However, Boron et al. showed no differences between progesterone gene polymorphisms (PROGINS and +331G/A) and the risk of preterm delivery [26]. Whereas Behram et al. showed a relationship between elevated levels of Il-22 and PROM [27]. A similar relationship was demonstrated for Aquaporin-9 [28].

The purpose of this study was to test the use of ICTP in the case of PROM. Fetal membranes are the outermost structure that protects the fetus from environmental factors. Their resistance to physical and chemical factors is largely due to the presence of type I collagen. Studies have shown that type I collagen is found in both the amnion (the compact layer, the fibroblast layer, the spongy layer) and in the chorion (the reticular layer) [2, 29].

Global apoptosis of the fetal membranes, the degradation of the intracellular matrix preceded by increased transcription of genes for metalloproteinases (MMPs) and their increased activity have been initially described in animal models [30]. In the case of human fetal membranes, the above-mentioned mechanisms of apoptosis and degradation occur mainly in the region of the fetal membranes near the inner os [31–33]. MMP-2 and MMP-9 cause the breakdown of type I collagen and one of the products of this process is ICTP, the carboxy-terminal telopeptide of type I collagen [3]. Studies conducted on the afterbirth from pregnancies that ended with physiological delivery showed that in the place of the physiological membrane rupture, the concentration of ICTP was three times higher than in the vicinity of the umbilical cord attachment [34]. In addition, studies to assess the concentration of ICTP in the amniotic fluid of women at increased risk of preterm premature rupture of

membranes (PPROM) showed that increased levels of ICTP were more frequent in patients who later experienced PPROM [35].

Our study shows that ICTP is present in cervical-vaginal fluid in pregnant women before physiological delivery. Conducting further studies in this direction may confirm the importance of collagen type I degradation in the mechanism of fetal membrane rupture. If the rupture of membranes in PPROM occurs via a similar molecular mechanism as in the case of physiological labor but much earlier before the maturity of the fetus, the identification of ICTP can help to recognize the beginning of the process of ROM before the onset of clinical signs of preterm labor and before physical interruption of their continuity. In the long term, this will enable the further delineation of the mechanisms of pathogenesis and the development of new tests to detect PPROM.

The strength of the research is its innovation. First, it describes the presence of a new marker in the mechanism of fetal membrane rupture. Moreover, it uses cervical-vaginal fluid, which is rarely used for research. This body fluid is taken non-invasively in a manner acceptable to patients, in contrast to the acquisition of amniotic fluid.

A weak point of the study is the small sample size. However, this study constitutes a beginning to research on ICTP.

CONCLUSIONS

In conclusion, ICTP is present in the cervical-vaginal fluid of pregnant women before physiological delivery. Further research is required to accurately assess ICTP as a marker of fetal membrane collagen degradation in the mechanisms of physiological delivery and premature membrane rupture.

Conflict of interest

All authors declare no conflict of interest.

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Table 1. Characterization of the study group with an additional division into subgroups depending on the concentration of ICTP

	Total	**Group 1**	**Group 2**	
	q2 (q1-q3)	**q2 (q1-q3)**	**q2 (q1-q3)**	**p**
Variable	**total**			
	21	**14**	**7**	
Mean ICTP value [ng/mL]	1.79 (1.02–2.12)	2.01 (1.81–2.18)	0.44 (0.31–0.97)	0.000
Age [years]	31.00 (28–34.00)	31.00 (28.5–33.75)	33.00 (28.5–36.50)	0.653
Week of gestational	39.29 (38.86–40.00)	39.29 (38.86–40.22)	39.00 (38.78–39.43)	0.764
Weight before pregnancy [kg]	60.00 (54–64.00)	57.50 (54–61.75)	60.00 (57–65.00)	0.409
Weight [kg]	71.00 (67–76.00)	70.50 (65–75.25)	75.00 (71–81.00)	0.166
BMI before pregnancy [kg/m^2]	21.61 (19.83–23.84)	21.55 (19.63–23.12)	22.86 (20.02–25.39)	0.287
Gravidity 1	7 (33.33)	6 (42.86)	1 (14.29)	

	2	6 (28.57)	3 (21.43)	3 (42.86)	0.376
	> = 3	8 (38.10)	5 (35.71)	3 (42.86)	
Parity	**1**	7 (33.33)	6 (42.86)	1 (14.29)	
	2	7 (33.33)	4 (28.57)	3 (42.86)	0.424
	> = 3	7 (33.33)	4 (28.57)	3 (42.86)	
Miscarriage	**No**	3 (14.29)	3 (21.43)	0 (0.00)	
	Yes	18 (85.71)	11 (78.57)	7 (100.00)	0.508
GBS vagina	**Negative**	15 (71.43)	10 (71.43)	5 (71.43)	
	Positive	3 (14.29)	2 (14.29)	1 (14.29)	1.000
	Not done	3 (14.29)	2 (14.29)	1 (14.29)	
GBS anal	**Negative**	13 (61.90)	9 (64.29)	4 (57.14)	
	Positive	3 (14.29)	2 (14.29)	1 (14.29)	0.933
	Not done	5 (23.81)	3 (21.43)	2 (28.57)	
Birth	**VD**	4 (19.05)	4 (28.57)	0 (0.00)	
	CD	17 (80.95)	10 (71.43)	7 (100.00)	0.326
Hypothyroidism	**No**	15 (71.43)	12 (85.71)	3 (42.86)	
	Yes	6 (28.57)	2 (14.29)	4 (57.14)	**0.124**
Gestational diabetes	**No**	19 (90.48)	13 (92.86)	6 (85.71)	
	Yes	2 (9.52)	1 (7.14)	1 (14.29)	1.000
Cervix dilatation	**0**	10 (47.62)	6 (42.86)	4 (57.14)	
[cm]	**0.5**	5 (23.81)	3 (21.43)	2 (28.57)	0.592
	> = 1	6 (28.57)	5 (35.71)	1 (14.29)	
Cervix	**Shortened**	14 (66.67)	12 (85.71)	2 (28.57)	
	long	7 (33.33)	2 (14.29)	5 (71.43)	**0.033**
First stage of delivery	**No**	13 (61.90)	7 (63.64)	6 (85.71)	
	Yes	5 (23.81)	4 (36.36)	1 (14.29)	0.631

CD — cesarean delivery; GBS — group B streptococcus; VD — vaginal delivery

Table 2. The concentration of the C-terminal telopeptide of type I collagen in the vaginal cervical fluid of pregnant women before physiological delivery

	Optimal Density			**ICTP concentration [ng/mL]**	
ID	**1st measurement**	**2nd measurement**	**Mean**	**Estimate (95% CI)**	
P1	1.6	1.7	1.7	519.7 (472.1–567.4)	
P2	1.8	1.9	1.9	616.2 (565.1–667.3)	
P3	1.7	NA	1.7	528.9 (480.9–576.8)	
P4	1.4	1.2	1.3	383.9 (340.5–427.2)	
P5	2.3	NA	2.3	806.2 (737–875.4)	ND
P6	1.7	NA	1.7	544.9 (496.5–593.4)	
P7	0.2	NA	0.2	43.7 (15.2–72.2)	
P8	2.3	NA	2.3	839 (764–914)	ND
P9	2.2	NA	2.2	762.6 (699.7–825.5)	
P10	0.2	0.5	0.4	88.5 (59.5–117.6)	
P11	1.8	2.1	2.0	657.5 (604.2–710.8)	
P12	1.0	NA	1.0	285 (246.2–323.9)	
P13	1.7	1.8	1.8	579.2 (529.6–628.8)	
P14	0.4	NA	0.4	103.9 (74.4–133.4)	
P15	2.0	NA	2.0	685.7 (630.5–740.9)	
P16	0.1	0.1	0.1	10 (–19.1–39.1)	
P17	1.0	0.9	0.9	248.5 (211.6–285.4)	
P18	2.3	2.0	2.2	756.6 (694.4–818.8)	
P19	2.3	2.0	2.1	730.3 (671.1–789.5)	
P20	1.9	2.1	2.0	664.1 (610.4–717.8)	
P21	2.5	NA	2.5	946.1 (845.5–1046.7)	ND

N/A — not available, the researchers didn’t manage to take the second sample.

ND — not detectable, the results were out of scale.

Figure 1. A flow chart of the recruitment of women

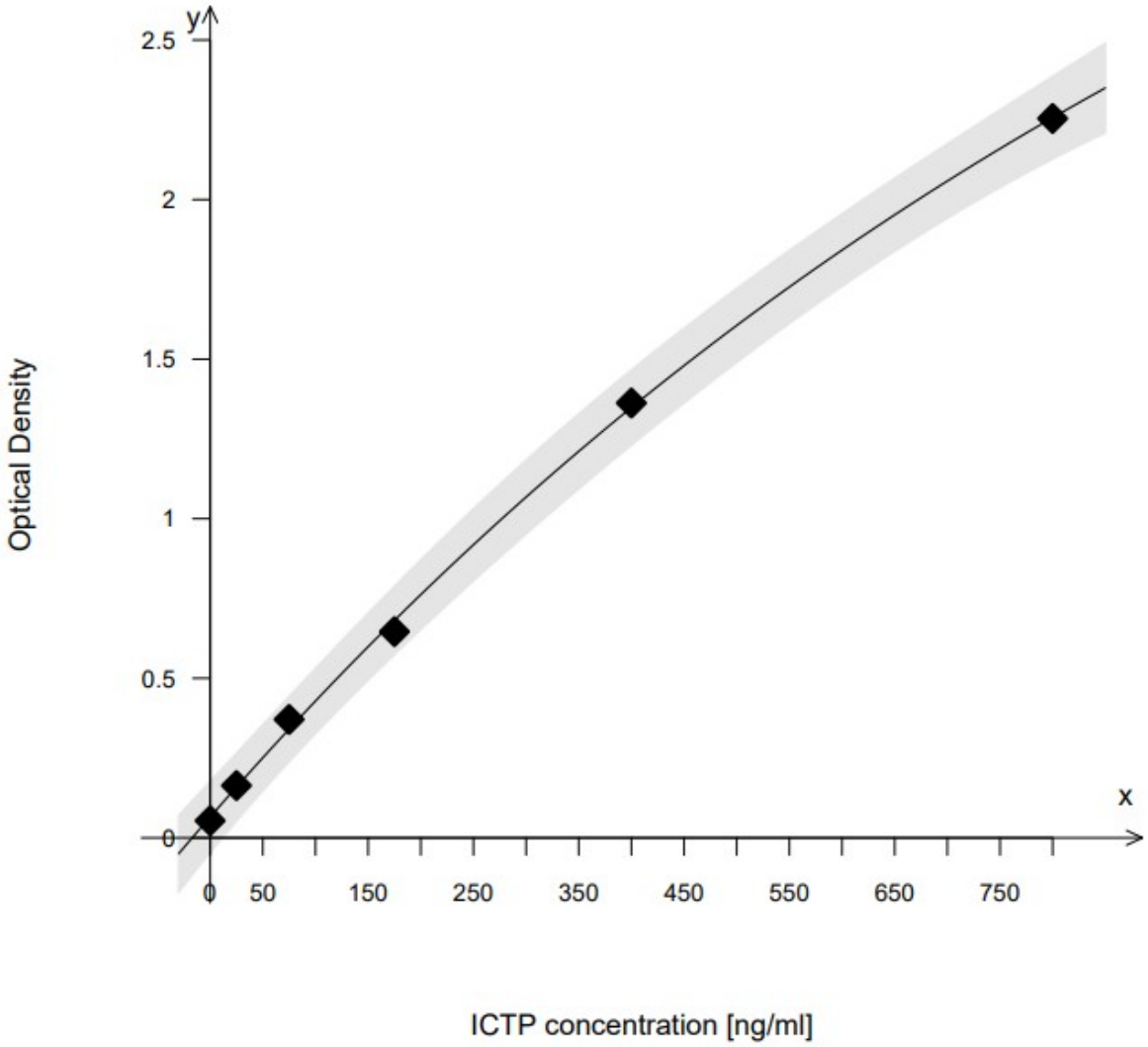

Figure 2. The standard curve generated by plotting the average optical density for each of the six standard concentrations and then used to determine the amount of ICTP in an unknown sample

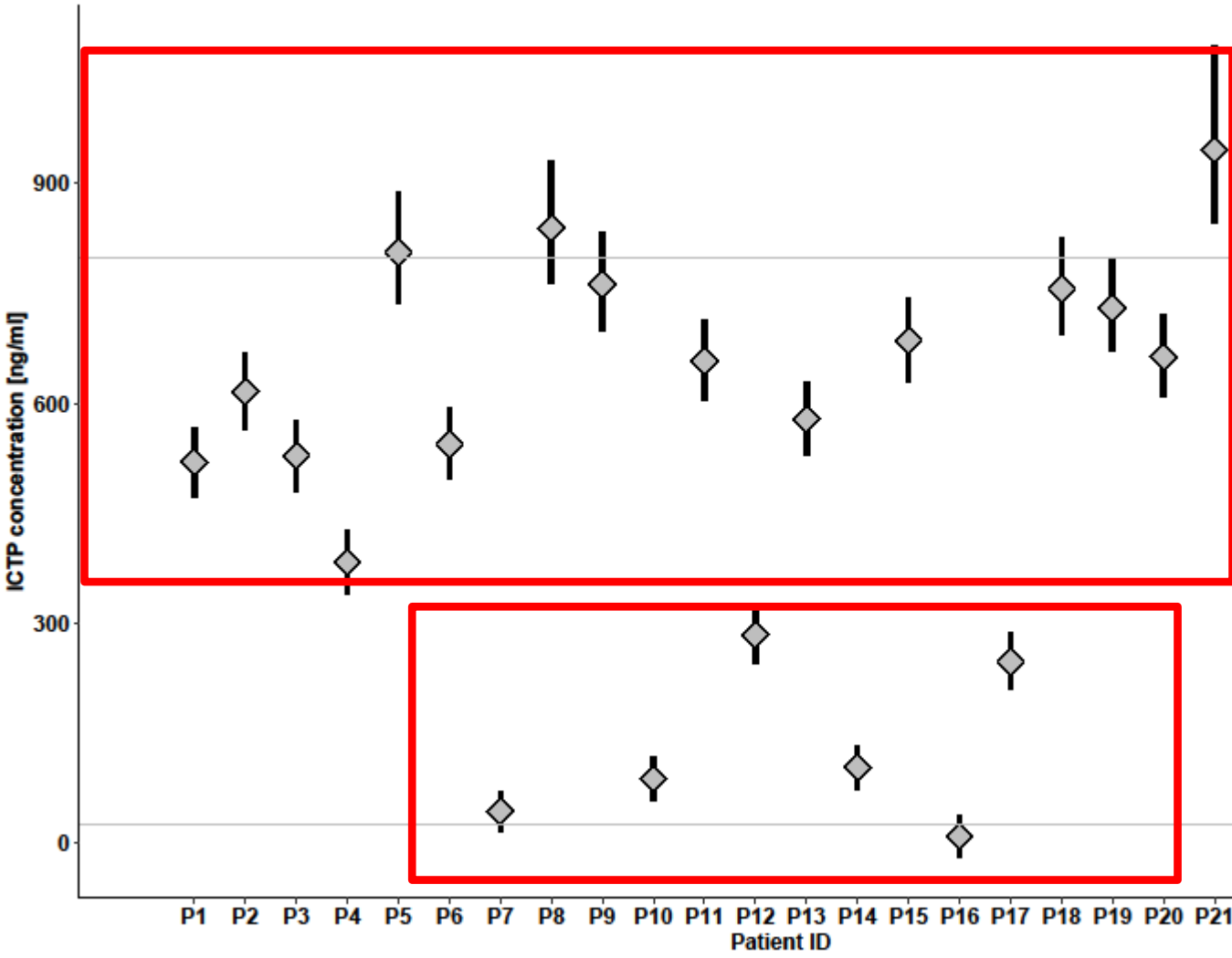

Figure 3. Estimated ICTP concentration among 21 patients included in analysis. Filled grey diamonds represent means, vertical lines reflect confidence intervals (95% CI). Horizontal grey lines depict the limits of the concentration range that the device can detect (25 ng/mL–800 ng/mL)