Clinical Research Article

A Reference Range for Plasma Levels of Inorganic Pyrophosphate in Children Using the ATP Sulfurylase Method

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Abstract

Purpose: Generalized arterial calcification of infancy, pseudoxanthoma elasticum, autosomal recessive hypophosphatemic rickets type 2, and hypophosphatasia are rare inherited disorders associated with altered plasma levels of inorganic pyrophosphate (PPi). In this study, we aimed to establish a reference range for plasma PPi in the pediatric population, which would be essential to support its use as a biomarker in children with mineralization disorders.

Methods: Plasma samples were collected from 200 children aged 1 day to 18 years who underwent blood testing for medical conditions not affecting plasma PPi levels. PPi was measured in proband plasma utilizing a validated adenosine triphosphate (ATP) sulfurylase method.

Results: The analytical sensitivity of the ATP sulfurylase assay consisted of 0.15 to 10 µM PPi. Inter- and intra-assay coefficients of variability on identical samples were below 10%. The standard range of PPi in the blood plasma of children and adolescents aged 0 to 18 years was calculated as 2.36 to 4.44 µM, with a median of 3.17 µM, with no difference between male and female probands. PPi plasma levels did not differ significantly in different pediatric age groups.

Main Conclusions: Our results yielded no noteworthy discrepancy to the reported standard range of plasma PPi in adults (2-5 µM). We propose the described ATP sulfurylase method as a diagnostic tool to measure PPi levels in plasma as a biomarker in the pediatric population.

Key Words: inorganic pyrophosphate, ATP sulfurylase assay, generalized arterial calcification of infancy, autosomal recessive hypophosphatemic rickets type 2, ENPP1, ABCC6
Extracellular inorganic pyrophosphate (PPi) is an important part of many molecular reactions in human metabolism. PPi consists of 2 inorganic phosphate (Pi) molecules combined through a hydrolysable ester bond. The synthesis of PPi is based on pyrophosphorolysis of nucleotide triphosphates, including adenosine triphosphate (ATP), which is synthesized intracellularly (1). Intracellular ATP synthesis is based on the oxidative phosphorylation of adenosine diphosphate in the inner mitochondrial membrane, glycolysis, and the tricarboxylic acid cycle (2).

Membrane-associated and partially soluble proteins function as key regulators and form a complex interaction to hold the extracellular PPi/Pi ratio constant (Fig. 1), which is essential to prevent ectopic mineralization (3-5). Pi is a key inhibitor of hydroxyapatite crystal growth (6) and 1 of the key endogenous inhibitors of ectopic calcification (3-5).

In humans, a low level of systemic PPi and, therefore, an off-balanced PPi/Pi ratio resulting in widespread arterial calcification have been found to be associated with mutations in the ENPP1 (ectonucleotide pyrophosphatase-phosphodiesterase 1) gene (7-9). Here, loss of ENPP1 function leads to a lack of sufficient supply of plasma PPi (Fig. 1).

Low levels of extracellular ATP could lead to physiologically low levels of PPi and adenosine monophosphate (10). This results from dysfunctional ATP-binding cassette subfamily C member 6 (ABCC6), which regulates the cellular export of ATP (Fig. 1) (11,12).

Another important player in PPi metabolism is tissue nonspecific alkaline phosphatase (TNAP). The enzyme may lose its function due to mutations within the ALPL (alkaline phosphatase, biomineralization associated) gene (13). PPi is not degraded to Pi, consequently leading to elevated plasma PPi levels (Fig. 1) (14,15).

The aforementioned metabolic anomalies are the cause of different disease patterns. Mutations in ENPP1 cause the rare human disease generalized arterial calcification of infancy (GACI) (7). PPi generation by ENPP1 is restricted and the PPi/Pi ratio is off balance. In patients with GACI, levels of systemic PPi measured in urine as well as in plasma PPi, are reported to be much lower—tending to be virtually zero—than in the published reference range (16). Affected patients experience calcification of the lamina elastica interna of arteries, which is associated with intimal proliferation leading to arterial stenoses (17). Mutations in ENPP1 have also been associated with autosomal recessive hypophosphatemic rickets (17).

**Figure 1.** Metabolism of extracellular inorganic pyrophosphate (PPi). Adenosine triphosphate (ATP) export is regulated by specific channels and transporters, including ATP-binding cassette subfamily C member 6 (ABCC6). Extracellular ATP is converted to adenosine monophosphate and PPi by ectonucleotide pyrophosphatase-phosphodiesterase 1 (ENPP1). Tissue nonspecific alkaline phosphatase (TNAP) degrades PPi to inorganic phosphate (Pi), while ecto-5’-prime-nucleotidase (CD73) degrades AMP to Pi and adenosine. Pi is transferred back intracellularly to synthesize ATP in the mitochondrial inner membrane through oxidative phosphorylation. Low levels of extracellular PPi can be caused by loss of function of ABCC6 or ENPP1. High levels of extracellular PPi can be caused by TNAP deficiency, as seen in hypophosphatasia.
Pseudoxanthoma elasticum (PXE), another rare disease, reflects a similar, although milder, phenotype. The disorder is characterized by yellow papules on the back and sides of the neck and in flexural areas and by angioid streaks in the retina, reflecting disruption of Bruch membrane and neovascular proliferation, which can lead to blindness (18). The skin becomes loose and wrinkled (19). In PXE, widespread arterial calcifications develop through adulthood (20). PXE is caused by a mutation in the ABCC6 gene (12,21,22). Recently, it has become clear that mutations in either ABCC6 or ENPP1 can cause both GACI and PXE (23,24).

Elevated PPi levels can be found in hypophosphatasia (14,15). Hypophosphatasia is caused by a deficiency of TNAP, which, under physiologic conditions, is necessary for the adequate mineralization of bones (25). Affected patients experience increased frailty and developmental disorders of the bone, calcification of kidneys, muscle weakness, and early loss of teeth (26). High plasma PPi levels arise from a dysfunctional enzymatic transformation reaction of PPi to Pi, performed by TNAP, caused by mutations in ALPL (13) and manifesting with lowered enzyme activity of serum alkaline phosphatase (27). Individuals with hypophosphatasia manifest the consequences of defective skeletal mineralization caused by very low levels of TNAP such as fractures, skeletal deformities with severe undermineralization of bones, rickets, and osteomalacia, along with early loss of deciduous teeth with intact roots, muscle weakness and pain, nephrocalcinosis, and vitamin B6-responsive seizures. There is considerable variability in expression in affected individuals both between and within families.

On the other hand, reduced extracellular accumulation of PPi, caused by increased alkaline phosphatase activity is part of the pathogenesis in the vascular calcification present in Hutchinson-Gilford progeria syndrome (28).

The first method to measure PPi in human samples was an isotope dilution method employing 32P-labeled PPi (14). Because a universal definition of a plasma PPi standard range did not exist, previous studies estimated a standard range by comparing diseased patients with healthy subjects. For example, a normal range of 1.7 to 5.5 μM was described by Armstrong et al (29) when comparing serum PPi in patients with osteogenesis imperfecta to healthy individuals. Altman et al, using the uridine diphosphate glucose (UDPG)-pyrophosphorylase method, reported a median of 3.5 μM PPi in human adult plasma (30). Cartier et al also defined a standard range in the context of establishing a slight variation of the UDPG-pyrophosphorylase method for PPi measurement. In that study, the plasma PPi level was 3.53 ± 0.19 μmol/L, including a range for this population (99% confidence limit) between 1.10 and 5.90 μmol/L (31). This method was picked up in further studies. At this point, the PPi concentration of plasma was found to be 2.72 ± 0.14 μM by Lust et al (32). Another normal plasma PPi concentration was defined as 2.18 μM with a range of 0.58 to 3.78 μM (95% confidence limits) by Ryan et al (33). The same research group introduced the yeast pyrophosphatase method and detected a normal range of 1.8 ± 0.06 μM (0.16-3.40 μmol/L) based on plasma samples from 94 healthy subjects (99% confidence limits) (34). This study interestingly reported lower levels compared with the others. All the previous studies employed plasma samples collected from adults and did not include pediatric samples.

Russell et al described an extracellular PPi range in human blood plasma between 2 and 5 μmol/L in adults (14). No statistically significant differences were observed between the sexes or different time of blood collection over the day. Furthermore, a consistent value of plasma PPi in individuals between the ages of 20 and 60 years had been demonstrated. Only a few younger study participants had higher PPi plasma levels. Considering the merely slightly increased PPi value in the small subgroup of pediatric participants, no certain statement about the correlation of age and PPi values in children could be drawn (14). Further studies have shown that the PPi/Pi ratio in children was 2 to 3 times higher than in adults when measured in urine samples (35), and it has been suggested that there is a difference in the plasma PPi standard range between adults and children (35).

In this study, we aim to establish a standard range for PPi in the blood plasma of children aged 0 to 18 years via the validated ATP sulfurylase method. In addition, we compare this standard range with individual PPi levels obtained from patients with GACI or hypophosphatasia. Establishing a standard range of plasma PPi should improve diagnostics and further treatment of children and adolescents with conditions related to altered plasma PPi levels.

**Materials and Methods**

**Study Participants**

The study population included 200 children and adolescents aged 0 to 18 years. To determine the statistical conclusion validity regarding a standard range, the number of study participants should be as high as possible. Based on the issue of taking blood samples from children and still keeping the study clinically feasible, the decision was made to evaluate a cohort of 200 participants in an outpatient setting of Muenster University Children’s Hospital. All samples were taken between 8 am and 2 pm from nonfasting children. We only collected blood from children who were already scheduled to undergo blood drawing during medical checkups.
Inclusion criteria were probands aged 0 to 18 years, visiting the outpatient clinics of Muenster University Children’s Hospital for various reasons. Most of the probands had been diagnosed with endocrine or metabolic disorders. Exclusion criterion was patients with diseases known to be dependent on PP, metabolism. Furthermore, probands with any disorder known to be involved in bone metabolism, patients with bone fractures, and patients who had surgeries including osteotomy were excluded.

In addition, we took blood samples from 1 individual with GACI and from an individual with mild hypophosphatasia, who manifested with dental cavities only and no bone phenotype and therefore did not receive enzyme replacement therapy. All caregivers received information about the study and signed a consent form. Participating children signed assent forms appropriate for their age, if possible. The study was approved by the Medical Ethics Committee of Muenster University (file number: 2017-575-f-S).

Blood Collection

Blood sampling was performed between 8 am and 2 pm in nonfasting individuals. After receipt of written informed consent, all 200 blood samples were collected in lithium-heparin tubes (1.3 or 7.5mL, depending on patient age) and immediately cooled on ice until further processing. Every sample was stored on ice no longer than 60 min. The entire processing time took less than 40 min. The time between centrifugation cycles was always about 2 or 3 min. In a first step, blood was centrifuged to separate the plasma (10 min at 1000 × g at 4°C). In a second step, plasma was depleted of components with high molecular weights, including platelets, by filtration through centrifugation with a Centrisart® I 300 kDa mass cutoff filter (Sartorius AG) to gain platelet-free plasma. During filtering of the plasma, the possibility of coagulation is high. To avoid this, we filtered only 1 mL of plasma per filter and spun at a maximum speed of 2200 × g for 20 min at 4°C. After this procedure, the remaining volume of platelet-free plasma ranged between 100 and 400 µL depending on how much full blood could be drawn from 1 proband beforehand. The platelet-free blood plasma sample was then frozen at −80°C in 100-µL aliquots.

Generation of Pyrophosphatase-treated Plasma

For human plasma, blood drawn from donors (everything at room temperature) was spun down at 3500 rpm for 15 min, and plasma was then transferred to a 15-mL Eppendorf AG tube. Yeast inorganic pyrophosphatase (PPase; New England Biolabs Inc) was added at 1:1000 plasma, mixed well by inverting 4 times, and incubated for 30 min at room temperature. Five hundred microliters of PPase-treated plasma was filtered through a Nanosep Centrifugal Device With Omega Membrane 30K (Pall Corp) at 13,200 × g for 5 to 10 min. The PPase-treated, filtered plasma was frozen at −80°C in 500 µL aliquots. A new stock was made every 2 months.

Assay Description and Validation

The assay utilized ATP sulfurylase to convert PP, to ATP, which was then detected by a luciferase/luciferin luminescence detection kit. The luminescent signal is directly proportional to the amount of ATP present; the assay is a semiquantitative assay (regulation 493.1256(d)(iii) of Clinical Laboratory Improvement Amendments of 1988). Target analyte of assay validation was PP, the specimens were human platelet-free plasma samples. The low limit of detection was 0.15 µM; the quantitative range was 0.15 to 10 µM. Normal ranges, determined by testing PP, levels of 50 healthy adult volunteers (50% male), were 1.43 to 7.40 µM (unpublished data). For validation and clinical testing, a standard curve was generated for each run by titrating measured amounts of PP, in PPase-treated pooled human plasma. The PP, standard curve was prepared by diluting powder PP, in water; thus, concentration was equal to 1 mM PP, final. One millimolar of PP, was diluted 1:100 in PPase-treated donor plasma and mixed well. Defined concentrations were achieved by serial 1:2 dilutions in PPase-treated donor plasma. A negative control of Hank’s balanced salt solution alone and PPase-treated pooled human plasma were included on each assay plate. Each concentration was run in triplicate.

PP, Assay Methodology

For PP, conversion, 35 µL of the reaction mixture [2.804 mL of water, 403.6 µL of 500 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid pH 7.4, 324.5 µL of 1 mM magnesium sulfate, 6.5 µL of 10 mM adenosine phosphosulfate, and 8.1 µL ATP sulfurylase enzyme] was added to each well of a 96-well polymerase chain reaction plate.

The assay was validated under Good Laboratory Practice, with inter- and intra-assay coefficient of variation (s/S) × 100 below 20%, receiver operating characteristic curve, and area under the curve >0.90 for all simulations. Analytical accuracy was established to be within 30% of previously measured samples.

For assay validation, the following was done in donor plasma: 1 mM PP, was diluted 1:100 in PPase-treated donor
plasma; defined concentrations were achieved by serial 1:2 dilutions in PPase-treated donor plasma. This was done in triplicate for each donor.

Per well, 5 µL of standard curve or untreated plasma sample was added in triplicate and mixed well. Samples were incubated at 37°C for 10 min followed by 90°C for 10 min and left at 4°C until the next step.

For final luminescence reaction, BacTiter-Glo kit (Promega Corp) was used. At room temperature, 20-µL BacTiter-Glo was mixed with 20-µL sample out of PP conversion and added to each well of white 96-well plates. The plates were incubated for 5 min and read on a plate reader. Each sample was analyzed in triplicate.

Statistics
The statistical analysis was created using SPSS software (IBM). Descriptive statistics in relation to age and PP value were prepared. The calculated range was set up by using the 5th and 95th percentiles. Furthermore, a linear regression including univariant distribution was applied to show the dependence of age and PP value. To determine possible discrepancies in sex distribution, the Whitney U test was applied.

The following were used to create the graphics/visual aids, including the box plots and histogram to visualize median and frequency: Prism (version 9.0.2 for Windows; GraphPad Software) and SPSS Statistics (version 26.0 for Windows, released 2019).

Results
Assay Validation
The analytical sensitivity consisted of 0.15 µM PPi. The accuracy was established to be within 20% of previously measured samples. Regarding reproducibility, inter- and intra-assay coefficients of variability on identical samples were 6.2% to 8.4% and 9.6%, respectively. The plasma PPi range was 0.15 to 10.0 µM; the average PPi concentration for all healthy adult volunteers was 2.8 µM, with an SD of 1.2 µM.

Study Results
From the total cohort of 200 pediatric probands, 193 blood plasma samples were analyzed with the validated ATP sulfurylase assay to define a standard range of PPi in platelet-free blood plasma derived from children and adolescents. Overall, 7 blood samples were excluded from the evaluation. Two samples were excluded because the probands were diagnosed after blood collection with diseases known to affect bone metabolism, which conflicted with our inclusion criteria. Four samples were excluded due to an error in the preanalytical process. One sample was excluded because of an error in the measurement workflow.

Of all study participants, a consistent age distribution from 0 to 18 years was established. Every age range between 0 and 18 years was represented, whereby there was a slight surplus of older children around the age of 15 (Fig. 2). The average age of the probands was 10.43 years (males = 10.95 years; females = 9.75 years).

The statistical evaluation of the 193 plasma samples produced a median value of 3.17 µM PPi, including the 5th to 95th percentiles. The standard value of PPi in the blood plasma of children and adolescents aged 0 to 18 years was calculated as 3.17 µM (range 2.36-4.44 µM) (Fig. 3A), with a slightly skewed distribution of 1.44 (Fig. 3B). A skewness between −1 and 1 would assume to be a normal distribution; therefore, nonparametric testing was used.

Individual plasma PPi levels show a tendency to decrease by 0.23 µM per year of life (Fig. 4). This applies for a P-value of 0.1 on regression analysis, which did not reach statistical significance. In our cohort, there was no statistically significant difference in PPi plasma levels at all ages.

The PPi values of 2 participants (45 and 47) were increased compared with all the other results, which were forming a uniform pattern (Fig. 4). No sources of error according to diseases, processing errors in preanalytics, or data analysis could be traced, which justified these levels. Both were considered statistical outliers.

The study population consisted of 85 males and 108 females. The different counts of male and female participants and difference in age distribution, as previously mentioned, could give a hint of influencing the median. To compare the sex distribution, the Whitney U test was used to identify contrasts in the distribution for continuous variables. This revealed a difference of 0.2 µM considering the median in both sexes (females 3.11 µM; males 3.31 µM), which did not reach statistical significance (Fig. 5). Hence, the difference had no impact on the calculated median.

Besides our cohort of 200 participants, we evaluated 1 individual with GACI and 1 individual with hypophosphatasia to compare and confirm the data, which had already been reported in the literature. The patient with GACI, carrying the ENPP1 variant c.2677G>T; p. E893* on both alleles, showed a considerably lower plasma PPi level (0.98 µM) than the standard range. The patient experiencing mild hypophosphatasia, carrying the monoallelic ALPL variant c.211C>T; p. R71C, showed an increased plasma PPi level (7.53 µM).
Discussion

This study aimed to establish a standard range of plasma PPi levels for infants, children, and adolescents who are not affected by a mineralization disorder. By these means, we aim to ease the identification of disorders that affect the PPi plasma level and facilitate a valid diagnosis as early as possible.

As shown in previous studies, a standard range of plasma PPi is defined as 2 to 5 µmol/L in adults aged 20 to 60 years using an isotope dilution method (14).

We define a standard range of PPi in the blood plasma of children and adolescents aged 0 to 18 years as 2.36 to 4.44 µM, with a median of 3.17 µM. This range was not dependent on age or sex in our pediatric cohort.

Due to the design of this study and some difficulties, which occur while collecting blood samples from children in general, more adolescents than infants or children participated in the study. All blood samples were taken during an already-occurring medical checkup. Consequently, the age of the population was bound by the age of the probands who were undergoing an examination during our study time. Resulting from this issue, a slight surplus of older children is ascertained. This could have influenced the observation of a slight difference in plasma PPi values between infants (slightly higher plasma PPi) and older children (slightly lower plasma PPi). However, regression analysis showed no impact on the median. The nonsignificant difference contributed so minimally that we did not consider to determine different standard ranges in different pediatric age groups. Russell et al set up this hypothesis earlier but was not able to reach a statistically significant result (14). Later, a decrease of the PPi concentration throughout a lifetime was
reported. Starting at the fifth decade of life, urinary PPi concentration was shown to decrease rapidly (35).

Comparing our results in children with PP i values and ranges measured in adults (14,31-34), there is a slight trend toward decreased PPi level in adults, not only in urine but also in plasma. Due to methodological differences among the different studies, the comparability should be critically assessed. The various procedures will be discussed later.

We found no significant difference between males and females, considering the median PPi level in both sexes. However, sound evidence of a different standard PPi range in male and female blood plasma would have required a higher and approximately equal number of participants in both test groups. Nevertheless, studies including more
test subjects already reported an equal plasma PPi level in adults of both sexes (14).

Furthermore, it has been reported that the PPi level in urine is dependent on the time of day and fasting state of the individual (36,37). Unfortunately, it was not possible in our study to influence the time of blood collection and make it a variable. All samples were taken between 8 AM and 2 PM from nonfasting children. Levels of PPi correlate to Pi, and PPi measured in urine and in plasma were lower in fasting probands and at night (36,37). In this context, the PPi excretion was found to be directly dependent on oral Pi intake (38).

To outline the discrepancy between a healthy level, described as a standard range, and patients with GACI and hypophosphatasia, this study included an individual with GACI and an individual with hypophosphatasia. The patient with hypophosphatasia had a significantly higher plasma PPi value of 7.53 µM. The patient with GACI had a considerably lower level of 0.98 µM. These levels are in line with previously reported results (14-16,39).

Our study results were obtained utilizing the ATP sulfurylase method. The main difficulty in establishing a standard range of PPi, whether in human plasma or urine, is the availability of a validated method, which is feasible simultaneously. Previously reported standard ranges had been established utilizing different methods. The first useable method to measure PPi plasma levels was the isotope dilution method [using 32P-labeled PPi] published by Fleisch and Bisaz (37) and modified by Russell et al (35). This radiometric assay showed major susceptibilities. The assay needs to separate PPi from other phosphate compounds by ion exchange chromatography (on an anion exchange resin) before measuring the specific radioactivity of the labeled PPi (14). Some groups modified this method
so that PP, after the separation from P, is measured according to acid hydrolysis. This separation is clearly the most complex part and technically most prone to disruptions; therefore, a large amount of sample is required (14). Difficulties also occur regarding the minimum measured quantity of blood and the complicated isotopic procedures; for that reason, Flodgaard et al introduced an isotope derivative method, which is performed with amounts as low as 10 pmol (40). Cartier et al developed the radiochemical method, which allowed a reduction of the spontaneous hydrolysis of PP, during separation (41). To avoid this complex separation in general, another assay based on the enzymatic reaction of UDPG-pyrophosphorylase was described. UDPG-pyrophosphorylase is coupled with other enzymes (phosphoglucomutase and glucose-6-phosphate dehydrogenase) to form nicotinamide adenine dinucleotide phosphate through reduction and phosphorylation. The nicotinamide adenine dinucleotide phosphate is then measured fluorometrically (42). This technique was further used in many studies, including variations (32, 43). Problems associated with this technique include a capillary microcuvette of long light path and complicated isotopic procedures (40). Later on, Cartier et al also used the UDPG method but recognized that the most essential aspect is to separate PP, from blood plasma to avoid the influence of inhibitory ions (31).

Silcox et al established the yeast pyrophosphate method, in which P, is extracted as a trimethylamine-molybdate precipitate and the remaining phosphomolybdate is removed, and then PP, is hydrolyzed by yeast PPase to measure PP, as P, later on (34). Another innovative solution to carry out the PP, measurement without complications is the multienzyme bioluminescent time-resolved pyrophosphate assay. Sun et al converted PP, to ATP through multiple enzyme reactions. ATP was used to measure the luminescence. The key challenges with this technique are (1) measuring the actual extracellular ATP and subtracting it from the end result and (2) the relatively time-consuming process required (44).

In the past, one of the main problems regarding the measurements of PP, levels in blood consisted of the use of thrombocyte-containing plasma. An important factor for the accuracy of extracellular PP, measurement is an exact preanalytical workflow. The possibility of bursting platelets in the obtained blood samples rises if the protocol is not strictly followed. Burst platelets set free intracellular ATP, which results in falsely high measurements of extracellular PP, also, PP, is released from platelets during coagulation (34). Using 3 different methods, Silcox et al observed that the PP, concentration in platelets is 800-fold higher than in blood plasma (45). Therefore, serum samples contained PP, levels that were 2 to 3 times higher than in plasma (46). Also, preanalytical problems may result from faults in timing. The time span between taking the blood sample and cooling it on ice, the storage time on ice, and the time while processing the blood in the centrifuge and between the 2 cycles of centrifugation should be kept as short as possible. To further prevent the platelets from bursting and from releasing intracellular PP, we used lithium-heparin tubes, because antithrombin III is activated by binding to heparin (47). Antithrombin prevents the formation of factor Xa out of factor X. Factor Xa converts prothrombin into thrombin. Thus, thrombin generation is blocked by heparin. Without this interruption of the coagulation cascade, the stimulation of thrombin would release about half of the PP, in the platelets (45).

We believe that it is essential to use thrombocyte-free plasma in the measurement and to strictly follow the preanalytical workflow to measure plasma PP, A comparison of our values to the ranges reported in the literature is hampered by the variety of utilized methods and PP, measurements in the presence of platelets. Although all previous assays showed a high degree of sensitivity, most of them are very complex to implement in daily clinical practice. With the ATP sulfurylase assay reported here and a strict preanalytical protocol, which includes plasma centrifugation through a 300 kDa mass cutoff filter and all samples analyzed in triplicate, we hope to overcome these limitations.

In summary, we established a standard range of PP, between 2.36 and 4.44 µM (5th–95th percentiles) in the blood plasma of children and adolescents aged 0 to 18 years by employing the ATP sulfurylase method. There were no significant differences between males and females or different pediatric age groups. The result yielded no noteworthy discrepancies in comparison with already-reported standard ranges in adults (2–5 µM/L). Establishing a standard plasma PP, range in infancy and childhood is of special relevance to support the use of plasma PP, as a biomarker when diagnosing rare inherited diseases, such as GACI, autosomal recessive hypophosphatemic rickets type 2, and hypophosphatasia. Moreover, plasma PP, may be used to monitor response to enzyme replacement therapy in patients with these diseases. In this respect, we propose the described ATP sulfurylase method as a valuable diagnostic tool.

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Additional Information

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