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Principal component and correlation analysis of biochemical and endocrine markers in a healthy pediatric population (CALIPER)

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ABSTRACT

Background: Reference intervals (i.e. normative ranges) established from a healthy reference population are essential to accurately interpret disease biomarkers. Biomarker concentration may partially depend on associations with other biomarkers due to various physiological and pathophysiological processes. In this study, a robust correlation analysis was performed to identify physiological biomarker associations in the healthy pediatric CALIPER cohort.

Methods: Population reference values for 35 biochemical and 20 fertility/endocrine markers were analyzed for correlations in all subjects, male adolescents, female adolescents, and young children. Associations between biomarkers were assessed by Spearman's rank correlation and a multivariate analysis technique, principal component analysis (PCA).

Results: Of 197, 90, 59, and 32 significant correlations between biochemical markers in all subjects, male adolescents, female adolescents, and children, respectively, 23, 19, 16, and 9 were moderately strong ($r > 0.5$ or $r < -0.5$). Of 98, 24, 33, and 16 significant correlations between fertility/endocrine markers in all subjects, male adolescents, female adolescents, and children, respectively, 17, 8, 11, and 5 were moderately strong. Results were agreeable between Spearman's rank method and PCA. In some cases, biomarker correlations differed between sexes.

Conclusions: Using PCA, this study provides for the first time an extensive analysis of circulating biomarker associations in a healthy pediatric cohort. These data can inform future studies of potential confounding factors or particular variables that should be considered in test result interpretation for specific diseases.

1. Introduction

The clinical laboratory provides objective data on biomarkers that directly aid in medical decisions for a wide range of clinical diseases. Physicians rely on laboratory test results for several aspects of clinical practice, including identifying symptoms and risk factors, diagnosing disease, determining appropriate treatment for a patient, and monitoring a patient's response to treatment. To correctly interpret laboratory test results, these values are compared to normative values (i.e. reference intervals), which are established based on a large, healthy reference population. The pediatric population is physiologically unique due to differences in physical size, organ maturity, body fluid compartments, immune and hormone responsiveness, nutrition, and metabolism [1]. Therefore, it is inappropriate to use adult reference intervals to interpret pediatric laboratory test results for several biomarkers. Rather, reference intervals established from a reference population comprised of healthy, pediatric subjects is critical to accurately interpret pediatric laboratory test results.

To address this gap, the Canadian Laboratory Initiative on Pediatric Reference Intervals (CALIPER) project recruited a large, healthy cohort of children and adolescents to establish a comprehensive database of pediatric reference intervals (www.caliperproject.ca) [2]. CALIPER has

Abbreviations: AFP, α-fetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; ASO, anti-streptolysin O; AST, aspartate aminotransferase; BMI, body mass index; CALIPER, Canadian Laboratory Initiative on Pediatric Reference intervals; CLSI, Clinical and Laboratory Standards Institute; CO_2, carbon dioxide; hsCRP, high-sensitivity C-reactive protein; C3, complement component 3; C4, complement component 4; FSH, follicle-stimulating hormone; FT3, free triiodothyronine; FT4, free thyroxine; GGT, gamma-glutamyl transferase; HDL-C, high-density lipoprotein cholesterol; iPTH, intact parathyroid hormone; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; LDH, lactate dehydrogenase; LH, luteinizing hormone; PCA, principal component analysis; SHBG, sex hormone binding globulin; TSH, thyroid-stimulating hormone; TT3, total triiodothyronine; TT4, total thyroxine

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established reference intervals specific for age, sex, and Tanner Stage [3–5] and has begun initiatives to examine the influence of ethnicity and body mass index (BMI) on biomarker concentrations. However, another significant covariate that can significantly alter physiological biomarker concentrations is in fact other biomarkers. Biomarker concentrations may correlate with each other due to various physiological and pathophysiological processes. Understanding associations between biomarkers may help identify potential confounding biomarkers that should be taken into consideration in future studies. For example, analyses to determine the prognostic potential of a particular biomarker may need to account for confounding biomarker concentrations [6]. Furthermore, associations between biomarkers may be helpful when defining exclusion criteria for reference interval establishment. For example, when establishing pediatric reference intervals for transferrin saturation, subjects with elevated C-reactive protein (CRP) were excluded, as the acute phase response down-regulates the hepatic synthesis of transferrin, subsequently decreasing total iron binding capacity (TIBC), which is used to calculate transferrin saturation [7]. Examining correlations between several biomarkers can lead to future studies of physiological processes underlying these biomarker relationships.

The present study used two different statistical methods to identify associations between biomarkers: pair-wise spearman correlations and principal component analysis (PCA). The multivariate analysis technique, PCA, reduces dimensionality of a large dataset to observe variation and strong patterns among original variables in a new, lower dimensional space without losing important information. PCA is commonly applied in chemical, pharmaceutical and medical research, as well as social sciences and other industries [8–11]. In the field of laboratory medicine, PCA has been used in method comparison studies, by using the composite of several methods as the “reference method” [12] and to optimize pre-analytical protocols for clinical proteomics studies [13]. This is the first study to use this statistical method, combined with pair-wise correlation analysis, to provide insight into the associations between several common serum biomarkers in a healthy pediatric population.

2. Methods

2.1. Participant recruitment and sample analysis

The CALIPER project has collected and analyzed serum samples from thousands of community children and adolescents, which is described in detail in previous CALIPER studies [3–5]. The present study examines laboratory data for 55 analytes previously generated by the CALIPER project. For the purpose of this study, only laboratory data from subjects aged 1– < 19 years were statistically analyzed. As PCA analysis requires data for all variables, participants < 1 year of age were excluded due to insufficient blood sample volume to analyze all tests for each subject. Only one assay for each analyte was examined in the present study: bromocresol purple assay for albumin, enzymatic assay for creatinine, and assays without pyridoxal phosphate for both alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Further information regarding analysis of routine chemistry markers, including analytical parameters and calibration/traceability information, are detailed in Colantonio et al. [3]. Further information regarding analysis of fertility hormones and endocrine markers is detailed in Konforte et al. and Bailey et al., respectively [4,5].

2.2. Statistical analysis

Correlations were determined between 35 routine biochemical markers, including: albumin, alkaline phosphatase (ALP), ALT, amylase, AST, direct bilirubin, total bilirubin, calcium, cholesterol, creatinine, gamma-glutamyl transferase (GGT), high-density lipoprotein cholesterol (HDL–C), iron, lactate dehydrogenase (LDH), lipase, magnesium, phosphate, total protein, triglycerides, uric acid, urea, anti-streptolysin O (ASO), carbon dioxide (CO_2), cholinesterase, apolipoprotein A1 (apoA1), apolipoprotein B (apoB), complement component 3 (C3), complement component 4 (C4), high-sensitivity C-reactive protein (hsCRP), haptoglobin, immunoglobulin A (IgA), immunoglobulin G (IgG), immunoglobulin M (IgM), prealbumin, and transferrin.

Correlations were also examined between 20 fertility hormones and endocrine markers, including: α-fetoprotein (AFP), cortisol, estradiol, ferritin, folate, follicle-stimulating hormone (FSH), free triiodothyronine (FT3), free thyroxine (FT4), homocysteine, luteinizing hormone (LH), intact parathyroid hormone (iPTH), progesterone, prolactin, sex hormone binding globulin (SHBG), testosterone, thyroid-stimulating hormone (TSH), total triiodothyronine (TT3), total thyroxine (TT4), vitamin B12, and 25(OH)-vitamin D.

Statistical analysis was performed on each dataset separately (i.e. routine chemistry and fertility/endocrine), as they were comprised of different reference individuals from the CALIPER cohort. Prior to performing statistical analysis, data were separated into the following groups; all subjects, male adolescents (10– < 19 years), female adolescents (10– < 19 years), and children (1– < 10 years of age). This allowed for the highlighting of age- and sex-related associations among the biomarkers. Subjects with missing values for any variable were omitted prior to analysis. For chemistry markers, the sample size for each subgroup was 458 (all subjects), 161 (male adolescents), 172 (female adolescents), and 125 (children). For fertility hormones and endocrine markers, the sample size for each subgroup was 284 (all subjects), 107 (male adolescents), 82 (female adolescents), and 95 (children).

2.2.1. Spearman's rank correlation analysis

Spearman's rank correlation analysis was performed to assess monotonous relationships between biomarkers using R software (R Version 3.5.1). Spearman's rank correlation coefficient, a non-parametric equivalent to the Pearson correlation coefficient to measure the statistical dependence between two variables, was used because the majority of biomarker concentrations were not normally distributed. Spearman's rank correlation coefficients (r) and *p*-values were determined between all chemistry markers and all fertility and endocrine markers in all subjects and each subgroup. Correlation coefficients (r) > 0.500 or < −0.500 were deemed moderately strong correlations [17]. All *p*-values were adjusted for multiple comparisons using the Bonferroni correction and statistical significance was determined as $\alpha < 0.05$. Correlation heatmaps were created to display the strength and direction of all biomarker correlations.

2.2.2. Principal component analysis (PCA)

PCA analysis was performed using Minitab package. PCA is a common statistical multivariate method that reduces the dimensionality of the data through transformation to a new set of variables, called principal components (PCs) with minimal loss of information [18–20]. The PCs are obtained as a linear combination of the original variables that preserve most of the data variance and generate a new low-dimensional space. The correlation between original variables (i.e. biomarker concentration and age) and the pattern of sample distribution can be explored through the space of the new components. By projecting individual subjects and original variables in the PC subspace, the score plot and the loading plot are extracted, respectively. Using the score plot for the first two components (i.e. PC1 and PC2), Mahalanobis distance was calculated for each data point and used to visually detect and remove multivariate outliers, indicating an unusual combination of two or more variables [21]. Mahalanobis distance is a measure of the distance between each individual data point and the center of the new PC space (i.e. PC1 and PC2), which can be thought of as the overall mean for multivariate data. PCA was used to analyze all chemistry markers and all fertility/endocrine markers in all subjects, male adolescents, female adolescents, and children. Loading plots of each dataset were created to visualize the relationships between the variables in the

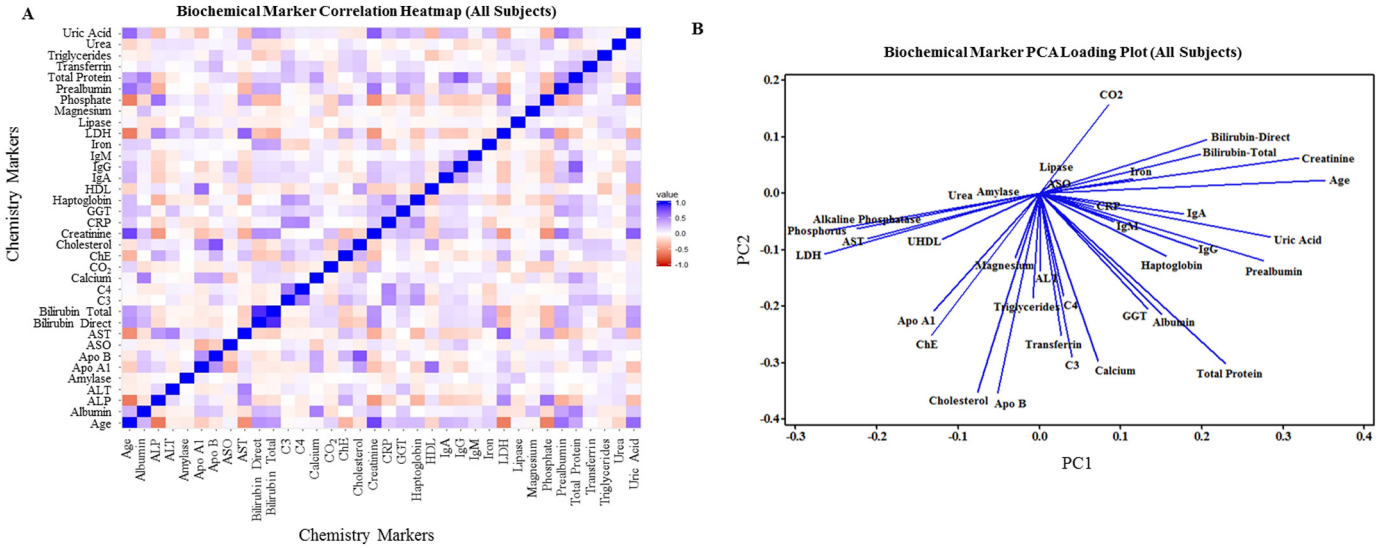

Fig. 1. Associations between 35 biochemical markers in the healthy pediatric CALIPER cohort aged 1– < 19 years. (A) Spearman correlation heatmap. The strength of the correlation between two variables is represented by the colour of the square at the intersection of those variables. Colours range from bright blue (strong positive correlation; i.e. $r = 1.0$) to bright red (strong negative correlation; i.e. $r = -1.0$). (B) Principal component analysis (PCA) loading plot. Positive associations between variables are identified when variables are clustered together (< 90° angle), with smaller angles representing stronger associations. Conversely, variables on opposite sides of the origin (i.e. approximately 180°) are negatively associated with each other. Variables at a 90° angle to each other are not associated. The length of the loading line for each variable represents the strength of the influence that variable has on the PCA model. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

space of the first two components (i.e. PC1 and PC2), which contain most of the variation in the data.

3. Results

Spearman's rank correlation coefficients were calculated and PCA was performed on two subsets of data: 35 routine chemistry markers (Figs. 1–3 and Supplemental Fig. 1) and 20 fertility hormones and endocrine markers (Figs. 4–6 and Supplemental Fig. 2). Using Mahalanobis distance, five and one outlier were visually detected and removed for routine chemistry markers and fertility/endocrine markers, respectively. The correlation heatmaps (Figs. 1–6A, Supplemental Figs. 1–2A) identify the strength of each correlation with colours ranging from bright blue ($r = 1.0$), indicative of a strong positive correlation, to bright red ($r = -1.0$), indicative of a strong negative correlation. The PCA loading plots (Figs. 1–6B, Supplemental Figs. 1–2B), showing the relationships between biomarkers in PC1 and PC2, depict positive associations as biomarkers clustered together (< 90° angle), with smaller angles representing stronger associations. Conversely, biomarkers on opposite sides of the origin (i.e. approximately 180°) are negatively

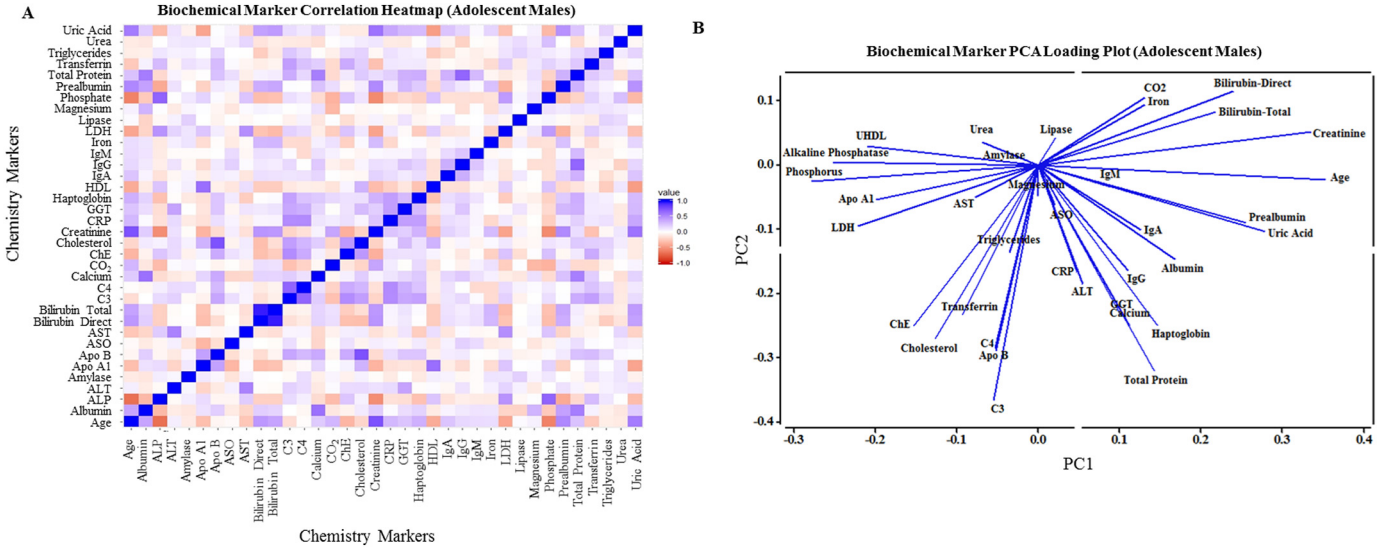

Fig. 2. Associations between 35 biochemical markers in adolescent males of the healthy pediatric CALIPER cohort aged 10– < 19 years. (A) Spearman correlation heatmap. The strength of the correlation between two variables is represented by the colour of the square at the intersection of those variables. Colours range from bright blue (strong positive correlation; i.e. $r = 1.0$) to bright red (strong negative correlation; i.e. $r = -1.0$). (B) Principal component analysis (PCA) loading plot. Positive associations between variables are identified when variables are clustered together (< 90° angle), with smaller angles representing stronger associations. Conversely, variables on opposite sides of the origin (i.e. approximately 180°) are negatively associated with each other. Variables at a 90° angle to each other are not associated. The length of the loading line for each variable represents the strength of the influence that variable has on the PCA model. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

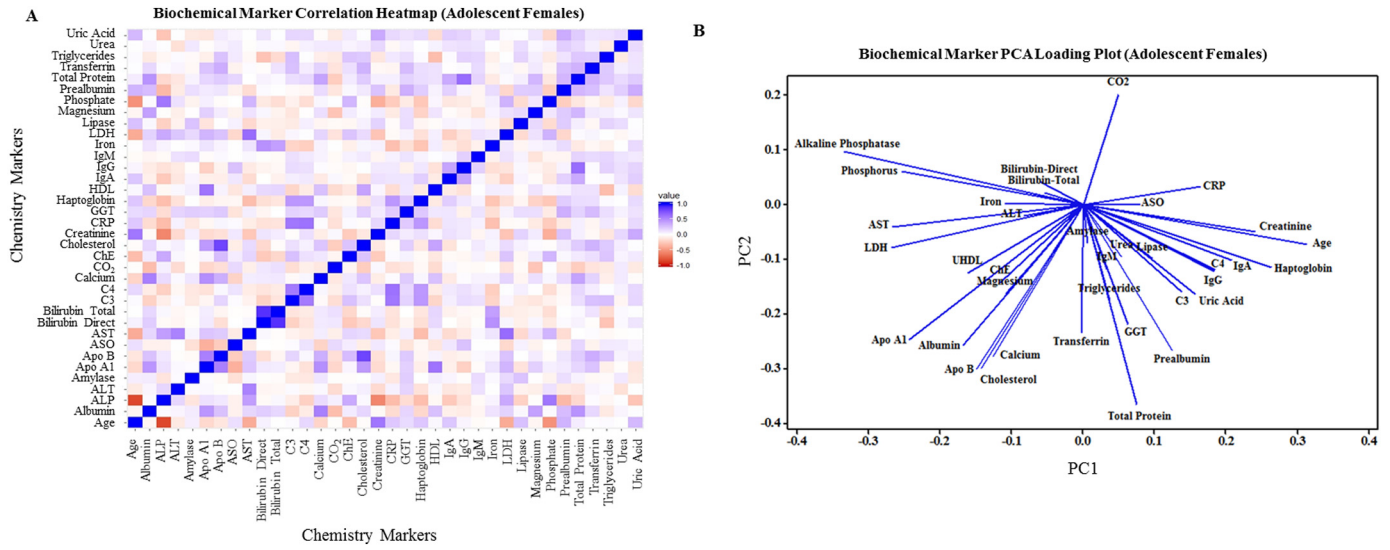

Fig. 3. Associations between 35 biochemical markers in adolescent females of the healthy pediatric CALIPER cohort aged 10- < 19 years. (A) Spearman correlation heatmap. The strength of the correlation between two variables is represented by the colour of the square at the intersection of those variables. Colours range from bright blue (strong positive correlation; i.e. $r = 1.0$) to bright red (strong negative correlation; i.e. $r = -1.0$). (B) Principal component analysis (PCA) loading plot. Positive associations between variables are identified when variables are clustered together (< 90° angle), with smaller angles representing stronger associations. Conversely, variables on opposite sides of the origin (i.e. approximately 180°) are negatively associated with each other. Variables at a 90° angle to each other are not associated. The length of the loading line for each variable represents the strength of the influence that variable has on the PCA model. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

associated. Biomarkers at a 90° angle to each other have no association. Furthermore, longer loading lines indicate the stronger influence of that biomarker on the PCA model. Overall, the results of the two statistical methods used to assess correlations between biomarkers strongly agreed. Supplemental Tables 1–4 provide all Spearman's rank correlation coefficients (r) and *p*-values (after Bonferroni correction) for moderately strong correlations between routine chemistry markers. Supplemental Tables 5–8 provide all Spearman's rank correlation coefficients (r) and p-values (after Bonferroni correction) for moderately strong correlations between fertility/endocrine markers. Supplemental Tables 9–10 provide the cumulative proportions of the explained variances for the first five extracted PCA components (i.e. PC1 – PC5) of chemistry markers and fertility and endocrine markers, respectively.

3.1. Routine chemistry marker correlations

In total, 595 pair-wise correlations were assessed between routine chemistry markers using Spearman's rank correlation method. Age was added as an additional variable, resulting in 630 total correlations

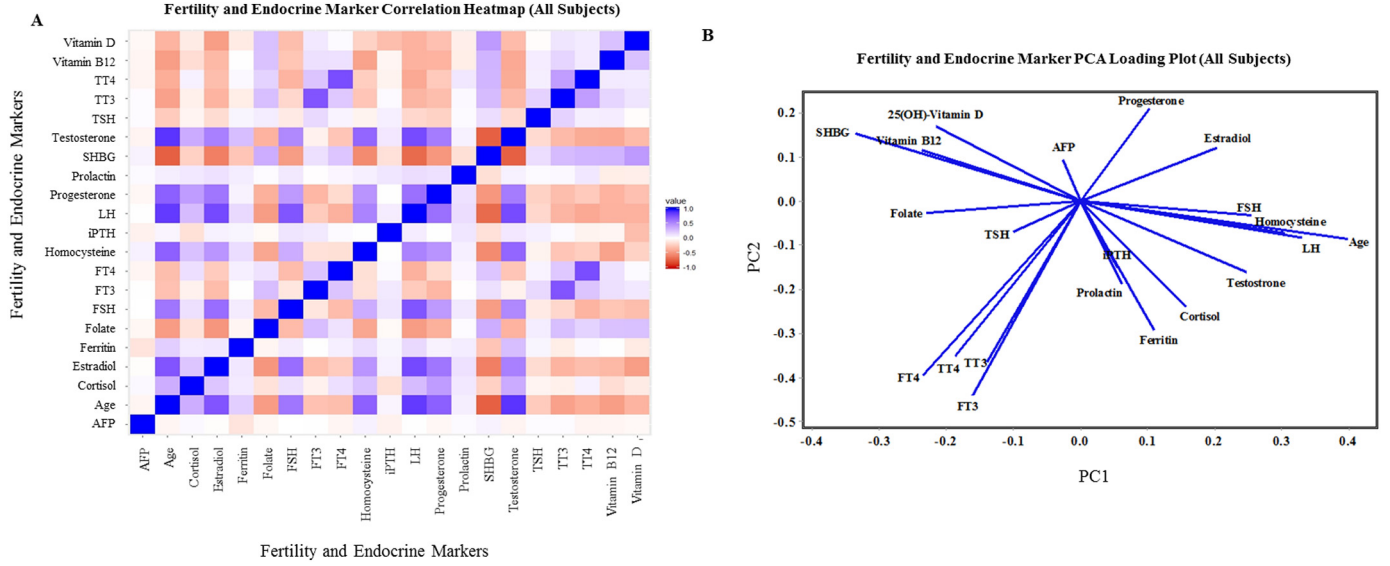

Fig. 4. Associations between 20 fertility and endocrine markers in the healthy pediatric CALIPER cohort aged 1– < 19 years. (A) Spearman correlation heatmap. The strength of the correlation between two variables is represented by the colour of the square at the intersection of those variables. Colours range from bright blue (strong positive correlation; i.e. $r = 1.0$) to bright red (strong negative correlation; i.e. $r = -1.0$). (B) Principal component analysis (PCA) loading plot. Positive associations between variables are identified when variables are clustered together (< 90° angle), with smaller angles representing stronger associations. Conversely, variables on opposite sides of the origin (i.e. approximately 180°) are negatively associated with each other. Variables at a 90° angle to each other are not associated. The length of the loading line for each variable represents the strength of the influence that variable has on the PCA model. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

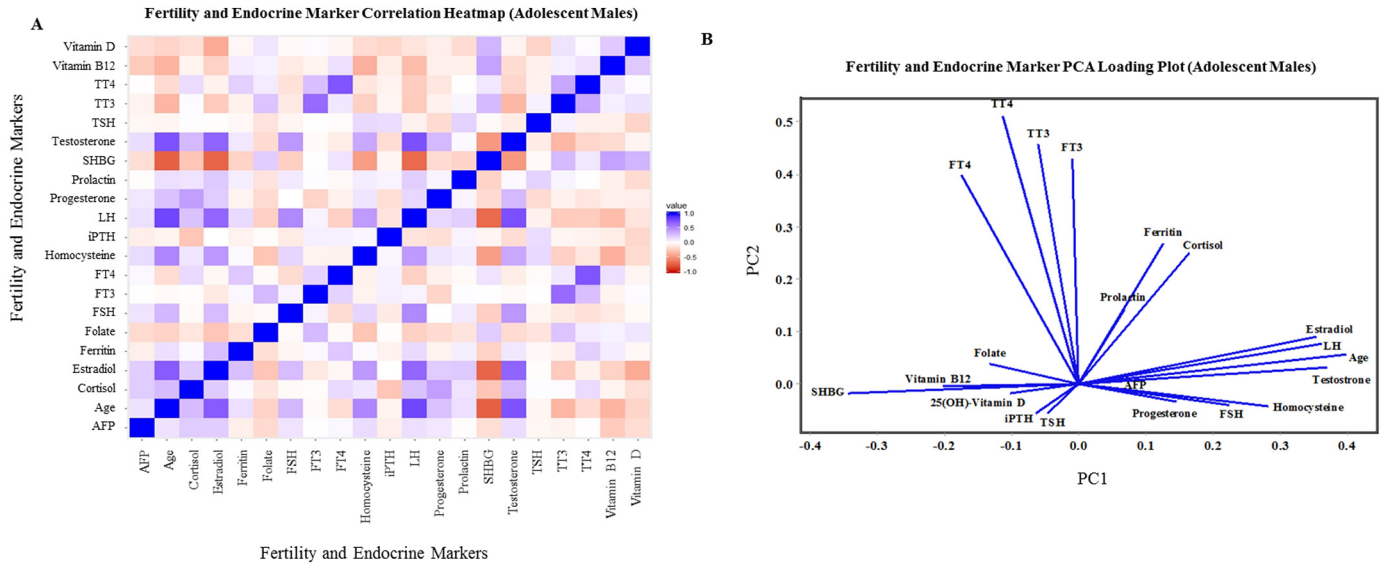

Fig. 5. Associations between 20 fertility and endocrine markers in adolescent males of the healthy pediatric CALIPER cohort aged 10– < 19 years. (A) Spearman correlation heatmap. The strength of the correlation between two variables is represented by the colour of the square at the intersection of those variables. Colours range from bright blue (strong positive correlation; i.e. r = 1.0) to bright red (strong negative correlation; i.e. r = −1.0). (B) Principal component analysis (PCA) loading plot. Positive associations between variables are identified when variables are clustered together (< 90° angle), with smaller angles representing stronger associations. Conversely, variables on opposite sides of the origin (i.e. approximately 180°) are negatively associated with each other. Variables at a 90° angle to each other are not associated. The length of the loading line for each variable represents the strength of the influence that variable has on the PCA model. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

analyzed. Of these, 197, 90, 59, and 32 correlations were statistically significant in all subjects, male adolescents, female adolescents, and children, respectively. Furthermore, 23, 19, 16, and 9 were moderately strong (i.e. $r > 0.5$ or $r < -0.5$) in the same respective groups. When all subjects were analyzed together, seven markers significantly correlated with age. Creatinine ($r = 0.80$), uric acid ($r = 0.64$), and prealbumin ($r = 0.62$) positively correlate with age, highlighted by the bright blue squares for these analytes against age in the correlation heatmap (Fig. 1A). Conversely, ALP ($r = -0.63$), LDH ($r = -0.61$), phosphate ($r = -0.60$), and AST ($r = -0.53$) decrease with age, highlighted by the bright red squares for these analytes against age in the correlation heatmap (Fig. 1A). The PCA loading plot in Fig. 1B also shows these relationships, as age clustered (small angle) with biomarkers showing a positive correlation (i.e. creatinine, uric acid, and prealbumin), and was on the opposite side of the origin (180° angle) in relation to those biomarkers which negatively correlated with age. Creatinine, phosphate, and ALP remained correlated with age in both male and female adolescents, when analyzed separately (Figs. 2 and 3,

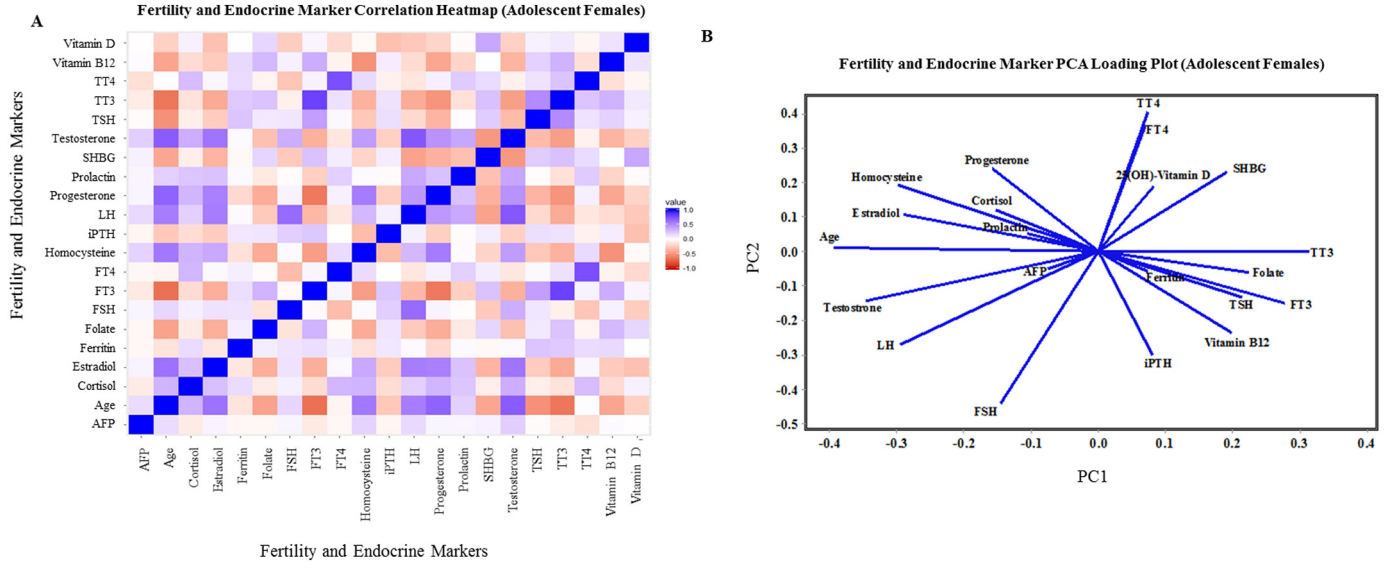

Fig. 6. Associations between 20 fertility and endocrine markers in adolescent females of the healthy pediatric CALIPER cohort aged 10– < 19 years. (A) Spearman correlation heatmap. The strength of the correlation between two variables is represented by the colour of the square at the intersection of those variables. Colours range from bright blue (strong positive correlation; i.e. r = 1.0) to bright red (strong negative correlation; i.e. r = −1.0). (B) Principal component analysis (PCA) loading plot. Positive associations between variables are identified when variables are clustered together (< 90° angle), with smaller angles representing stronger associations. Conversely, variables on opposite sides of the origin (i.e. approximately 180°) are negatively associated with each other. Variables at a 90° angle to each other are not associated. The length of the loading line for each variable represents the strength of the influence that variable has on the PCA model. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

respectively). However, uric acid and prealbumin were only found to be correlated with age in male adolescents. In children, age only significantly correlated with creatinine ($r = 0.57$) (Supplemental Fig. 1).

In total, 16 moderately strong and significant correlations were identified between routine chemistry markers when all subjects were analyzed together (Fig. 1**,** Supplemental Table 1). When males and females were analyzed separately, there were 14 and 13 moderately strong and significant correlations (Figs. 2–3, Supplemental Tables 2–3). When children were analyzed separately, there were only eight moderately strong and significant correlations (Supplemental Fig. 1, Supplemental Table 4). Several expected associations between biomarkers were seen, including among lipids/lipoproteins (e.g. apoB and cholesterol, apoA1 and HDL–C), proteins (e.g. IgG and total protein, albumin and calcium), inflammatory markers (e.g. C3, C4, CRP), and liver markers (e.g. ALT, AST, LDH). These associations were apparent from both the heatmaps and PCA loading plots among all subjects, as well as male and female adolescents, when analyzed separately. Most associations were less apparent in children, with only correlations between apoB and cholesterol, apoA1 and HDL–C, and IgG and total protein remaining moderately strong. Analyzing biomarker associations in smaller subgroups showed that some are dependent on age and/or sex. For example, creatinine negatively correlated with phosphate in males, but not females. Conversely, creatinine positively correlated with uric acid and prealbumin in males, but not females. Furthermore, creatinine and ALP negatively correlated in male and female adolescents, however, this association was not present in children. Lastly, haptoglobin correlated with C3, C4, and CRP in children, but these relationships were not seen in adolescents or all subjects analyzed together.

3.2. Fertility hormones and endocrine marker correlations

Between fertility hormones and endocrine markers, 190 pair-wise correlations were assessed using Spearman's rank correlation method. A total of 210 correlations were analyzed, with age included as an additional variable. Of these, 98, 24, 33, and 16 correlations were statistically significant in all subjects, male adolescents, female adolescents, and children, respectively. Furthermore, 24, 13, 19, and 7 were moderately strong (i.e. $r > 0.5$ or $r < -0.5$) in the same respective groups. When all subjects were analyzed together, seven markers significantly correlated with age. Testosterone ($r = 0.87$), LH ($r = 0.85$), estradiol ($r = 0.74$), progesterone ($r = 0.69$), homocysteine ($r = 0.69$), and FSH ($r = 0.60$) increase with age, highlighted by the bright blue squares for these analytes against age in the correlation heatmap (Fig. 4A**)**. On the other hand, SHBG ($r = -0.74$) decreases with age, highlighted by the bright red squares for these analytes against age in the correlation heatmap (Fig. 4A**)**. The PCA loading plot in Fig. 4B also shows this relationship with age clustering (small angle) with those showing a positive correlation, and age on the opposite side of the origin (almost 180° angle) from those showing a negative correlation. These associations remained apparent when adolescent males and females were analyzed separately, with the exception of FSH. Additionally, age was not correlated with progesterone in males, and SHBG was not correlated with age in females (Figs. 5–6). Thyroid hormones (including FT3, TT3, and TSH) were negatively correlated with age only in adolescent females, evident from both the correlation heatmaps and PCA loading plots **(**Figs. 6**)**. In children, age only significantly correlated with progesterone ($r = 0.74$) and testosterone ($r = 0.74$) (Supplemental Fig. 2).

In total, 17 moderately strong and significant correlations were identified when all subjects were analyzed together (Fig. 4). When male and female adolescents were analyzed separately, there were eight and 11 moderately strong and significant correlations (Figs. 5–6). When children were analyzed separately, there were only five moderately strong and significant correlations. Several expected associations between biomarkers were seen including among LH, FSH, testosterone, estradiol, and progesterone. These associations were apparent from both the heatmaps and PCA loading plots among all subjects, as well as male and female adolescents, when analyzed separately. Analyzing fertility and endocrine marker associations in smaller subgroups showed that some of these associations are dependent on age and/or sex. Negative correlations between homocysteine and vitamin B12, as well as between FT3 and progesterone were only apparent in adolescent females. Furthermore, SHBG was negatively correlated with estradiol and LH only in adolescent males. Evident from both the correlation heatmaps and PCA loading plots, associations between thyroid hormones differed between adolescent males and females (Figs. 5–6). In males, FT3, TT3, FT4, and TT4 were all positively correlated with each other (blue squares), but were not correlated with TSH (white squares) **(**Fig. 5A**)**. This was also evident in the PCA loading plots where FT4, TT4, FT3, and FT3 all cluster together **(**Fig. 5B**)**, indicating a positive association between them. These four thyroid hormones were also 90° to TSH, indicating no association with TSH. In contrast, the PCA loading plot for females **(**Fig. 6B**)** showed TT3, FT3, and TSH clustering, and at 90° to FT4 and TT4, indicating a positive association between TSH, TT3, and FT3, but no association between these markers and FT4 and TT4. This is further supported by the correlation heatmap **(**Fig. 6A**)**, with no correlations between FT3, TT3, TSH and FT4, TT4 (white squares), but a positive correlation between FT3, TT3 and TSH (blue squares).

4. Discussion

This study describes an application of PCA in the field of laboratory medicine by identifying numerous associations between 35 routine chemistry biomarkers and between 20 fertility hormones and endocrine markers in a large, healthy pediatric population. PCA analysis, which transforms the dataset into a new, lower-dimensional subspace to subsequently determine associations among original variables, was displayed using loading plots of the first and second PCs (i.e. PC1 and PC2). This method reduces the dimensionality of datasets to improve data interpretation without losing information. Positive and negative associations can also be easily observed from the loading plots with clustering biomarkers (small angles) indicating positive associations and biomarkers on opposite sides of the origin (180° angle) indicating negative associations. In addition to PCA analysis, relationships between biomarkers were also examined using Spearman's correlation rank method. Spearman's correlations were displayed using correlation heatmaps, with positive and negative correlations easily observed as blue and red squares, respectively, at the intersection of two biomarkers. Establishing associations between biomarkers, as well as differences in these associations between age and sex, identifies factors that may need to be considered when interpreting biomarker concentration, adjusting for confounding factors in clinical research studies, as well as selecting variables to consider when developing algorithms for risk assessment and/or diagnosis in future studies of specific diseases.

The effect of age was determined by Spearman correlation analysis and by comparing correlations between children and adolescents. For example, of seven chemistry markers that significantly correlated (positive or negative) with age across all subjects, only creatinine remained positively correlated with age in each subgroup (i.e. male adolescents, female adolescents, and children), indicating that creatinine increases with age throughout childhood and adolescence. Conversely, phosphate and ALP only remained negatively correlated with age in adolescents, but not in children, suggesting phosphate and ALP concentrations remain consistent throughout childhood, but decrease throughout adolescence. As the majority (i.e. 77–89%) of total ALP is of bone origin [22], the age-related decrease in ALP is expected after puberty due to cessation of bone growth [3,23]. Lastly, uric acid and prealbumin only remained positively correlated with age in adolescent males, perhaps due to higher muscle mass in males, as uric acid increases as a result of protein degradation. Age-related changes in biomarker concentrations

are typically examined using statistical methods, which simply compare the mean and variance between two subsequent age groups [24]. When two adjacent age groups are deemed to be significantly different, both statistically and clinically, reference intervals are often partitioned, meaning a separate reference interval will be created for each of these age groups. However, analysis of correlations between age and biomarker concentration, while treating age as a continuous variables, rather than a dichotomous variable, allows age-related trends to be identified, as shown in the present study. These results also further highlight the potential benefit of establishing continuous reference intervals, rather than partitioned reference intervals, to better reflect the dynamic age-related trend in biomarker concentration [25]. Using data from the CALIPER cohort, our research group is currently establishing continuous reference intervals and comparing their clinical utility to previously established partitioned reference intervals. Furthermore, when analyzing biomarker concentration differences between two sex groups without taking age into consideration, only absolute concentration differences will be identified. However, it is shown here that age-related concentration changes, not only absolute concentration, can be sex-specific (i.e. the average biomarker concentration may be comparable between sexes, yet the change in concentration with age may differ between sexes).

Several associations reported between chemistry markers and fertility/endocrine markers were expected based on known physiological processes. Among chemistry markers, these include lipids/lipoproteins (i.e. apoB and cholesterol, apoA1 and HDL–C), proteins and electrolytes (i.e. IgG and total protein, albumin and calcium), inflammatory markers (C3, C4, CRP), and liver markers (i.e. ALT, AST, LDH). For example, a positive correlation between apoB and cholesterol is expected, as apoB is the primary structural protein of several lipoproteins (e.g. very-low-density lipoproteins (VLDL) and low-density lipoproteins (LDL)), which carry lipids, including cholesterol, to cells within body tissues. Similarly, the significant positive correlation between apoA1 and HDL-C is expected as apoA1 is the major apolipoprotein of HDL particles. Among fertility and endocrine markers, expected associations among LH, FSH, testosterone, estradiol, and progesterone were also identified. These associations were apparent from both correlation heatmaps and PCA loading plots among all subjects, as well as in male and female adolescents, when analyzed separately. Thus, the findings reported here demonstrate the comparability between the two statistical methods used to determine biomarker associations, as well as the validity of these methods as well-known biomarker associations were identified.

In addition to expected biomarker associations, several novel associations were identified, particularly in regard to sex differences. In the present study, we were able to observe sex differences in biomarker associations, rather than simply biomarker concentration. For example, creatinine is significantly negatively correlated with phosphate and positively correlated with uric acid and prealbumin in adolescent males, but not in adolescent females. The stronger associations between creatinine and other biochemical markers in males compared to females is evident by both the correlation heatmaps (i.e. more bright blue and red squares for male adolescents) (Figs. 2A, 3A) and loading plot (i.e. longer line for creatinine for male adolescents) (Figs. 2B, 3B). A sex difference in creatinine concentration has been established in the pediatric population [3], explained by the fact that creatinine is produced from the breakdown of creatine in muscles and males have higher muscle mass on average than females. However, this study now identifies that creatinine not only differs between sexes in absolute concentration, but also associates with biomarkers differently. Sex-specific associations between creatinine and other biomarkers in healthy states can be important when interpreting creatinine values. For example, serum creatinine may be falsely increased above the reference range in individuals with high muscle mass or may remain within the reference range despite renal impairment, due to low muscle mass [26]. While muscle mass has been extensively studied in the context of creatinine, associations with other biomarkers may have a similar impact. It is also of interest to determine if associations between creatinine and other biomarkers change in diseased states and if this has implications for estimating glomerular filtration rate.

Significant correlations were also found among thyroid hormones, but to a greater extent in female compared to male adolescents. Firstly, TSH, TT3, and FT3 all negatively correlated with age only in adolescent females. Furthermore, adolescent females, but not males, exhibited significant correlations between TSH, TT3, and FT3 (Fig. 6). A recent study by Li et al. examined correlations between TSH and TT3, TT4, FT3, and FT4 in patients with hyperthyroidism, hypothyroidism, as well as healthy adults. In healthy adults TSH had a weak negative correlation with FT4 and TT4, but no correlation with FT3 or TT3 [27]. In contrast, the present study found that when analyzing all pediatric subjects together, TSH did not significantly correlate with any thyroid hormones. Differences between studies may be due to the age, sex, and ethnicity of the populations utilized. Li et al. utilized a population of Chinese adults aged 20–60 years, with more than twice as many females as males. Unlike the present study, Li et al. did not examine correlations among TT3, FT3, TT4, and FT4, or differences in thyroid hormone correlations between sexes or in different age groups. Li et al. noted that correlations between thyroid hormones differ between a healthy population and a population with hyperthyroidism and hypothyroidism [27], supporting the fact that understanding biomarker associations in a healthy population can aid in laboratory assessment of patients.

This study provides, for the first time, an extensive analysis of associations between numerous serum biomarkers including routine biochemical markers, fertility hormones, and endocrine markers in a healthy pediatric population. Several sex differences in associations between biomarkers were evident through both Spearman's rank correlations and PCA analysis. The relatively few strong correlations identified suggests the strength of routinely tested analytes, as they often provide independent information. Further studies are warranted to validate the reproducibility of these findings and further investigate the clinical relevance of observed sex differences. A limitation of this study is the absence of diseased cohorts, as the inclusion of such individuals would provide the ability to develop diagnostic algorithms. The breadth of analytes examined in this study, however, provide a foundation for future studies to assess changes in biomarker associations in specific diseased states. Overall, this study describes an application of PCA in clinical chemistry and identifies associations between biomarkers under physiological conditions in the pediatric population.

Conflict of interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinbiochem.2019.02.004.

References

[1] B. Jung, K. Adeli, Clinical laboratory reference intervals in pediatrics: the CALIPER initiative, Clin. Biochem. 42 (16–17) (2009 Nov) 1589–1595.
[2] K. Adeli, Closing the gaps in pediatric reference intervals: the CALIPER initiative, Clin. Biochem. 44 (7) (2011 May) 480–482.

[3] D.A. Colantonio, L. Kyriakopoulou, M.K. Chan, C.H. Daly, D. Brinc, A.A. Venner, et al., Closing the gaps in pediatric laboratory reference intervals: a CALIPER database of 40 biochemical markers in a healthy and multiethnic population of children, Clin. Chem. 58 (5) (2012 May 1) 854–868.
[4] D. Bailey, D. Colantonio, L. Kyriakopoulou, A.H. Cohen, M.K. Chan, D. Armbruster, et al., Marked biological variance in endocrine and biochemical markers in childhood: establishment of pediatric reference intervals using healthy community children from the CALIPER cohort, Clin. Chem. 59 (9) (2013 Sep) 1393–1405.
[5] D. Konforte, J.L. Shea, L. Kyriakopoulou, D. Colantonio, A.H. Cohen, J. Shaw, et al., Complex biological pattern of fertility hormones in children and adolescents: a study of healthy children from the CALIPER cohort and establishment of pediatric reference intervals, Clin. Chem. 59 (8) (2013 Aug) 1215–1227.
[6] M.A. Pourhoseingholi, A.R. Baghestani, M. Vahedi, How to control confounding effects by statistical analysis, Gastroenterol. Hepatol. Bed Bench. 5 (2) (2012) 79–83.
[7] V. Higgins, M.K. Chan, K. Adeli, Pediatric reference intervals for transferrin saturation in the CALIPER cohort, eJIFCC. 28 (1) (2017 Feb) 77–84.
[8] D.-S. Zhao, L.-L. Jiang, Y.-X. Fan, L.-C. Dong, J. Ma, X. Dong, et al., Identification of urine tauro-β-muricholic acid as a promising biomarker in Polygoni Multiflori radix-induced hepatotoxicity by targeted metabolomics of bile acids, Food Chem. Toxicol. Int. J. Publ. Br. Ind. Biol. Res. Assoc. (2017 Feb) 24.
[9] P.J. Markiewicz, J.C. Matthews, J. Declerck, K. Herholz, Robustness of correlations between PCA of FDG-PET scans and biological variables in healthy and demented subjects, NeuroImage 56 (2) (2011 May 15) 782–787.
[10] D.A. Jackson, Y. Chen, Robust principal component analysis and outlier detection with ecological data, Environmetrics 15 (2004) 129–139.
[11] X. Zhang, Q. Gong, Correlation between the frequency difference limen and an index based on principal component analysis of the frequency-following response of normal hearing listeners, Hear. Res. 344 (2017 Feb) 255–264.
[12] R.N. Carey, S. Wold, J.O. Westgard, Principal component analysis: an alternative to "referee" methods in method comparison studies, Anal. Chem. 47 (11) (1975 Sep) 1824–1829.
[13] J.F. Timms, E. Arslan-Low, A. Gentry-Maharaj, Z. Luo, D. T'Jampens, V.N. Podust, et al., Preanalytic influence of sample handling on SELDI-TOF serum protein profiles, Clin. Chem. 53 (4) (2007 Apr) 645–656.
[17] M.M. Mukaka, Statistics corner: a guide to appropriate use of correlation coefficient in medical research, Malawi Med. J. J. Med. Assoc. Malawi. 24 (3) (2012 Sep) 69–71.
[18] K.Y. Yeung, W.L. Ruzzo, Principal component analysis for clustering gene expression data, Bioinforma. Oxf. Engl. 17 (9) (2001 Sep) 763–774.
[19] J.B. Lhoest, M.S. Wagner, C.D. Tidwell, D.G. Castner, Characterization of adsorbed protein films by time of flight secondary ion mass spectrometry, J. Biomed. Mater. Res. 57 (3) (2001 Dec 5) 432–440.
[20] I.T. Jolliffe, Principal Component Analysis, Springer Series in Statistics, 2nd ed, Springer-Verlag New York, Inc., 2002.
[21] R. De Maesschalck, D. Jouan-Rimbaud, D.L. Massart, The Mahalanobis distance, Chemom. Intell. Lab. Syst. 50 (1) (2000 Jan) 1–18.
[22] S. Turan, B. Topcu, İ. Gökçe, T. Güran, Z. Atay, A. Omar, et al., Serum alkaline phosphatase levels in healthy children and evaluation of alkaline phosphatase z-scores in different types of rickets, J. Clin. Res. Pediatr. Endocrinol. 3 (1) (2011) 7–11.
[23] K. Adeli, V. Higgins, M. Nieuwesteeg, J.E. Raizman, Y. Chen, S.L. Wong, et al., Biochemical marker reference values across pediatric, adult, and geriatric ages: establishment of robust pediatric and adult reference intervals on the basis of the Canadian health measures survey, Clin. Chem. 61 (8) (2015 Aug) 1049–1062.
[24] E.K. Harris, J.C. Boyd, On dividing reference data into subgroups to produce separate reference ranges, Clin. Chem. 36 (2) (1990 Feb) 265–270.
[25] J. Bjerner, Age-dependent biochemical quantities: an approach for calculating reference intervals, Scand. J. Clin. Lab. Invest. 67 (7) (2007) 707–722.
[26] A.C. Baxmann, M.S. Ahmed, N.C. Marques, V.B. Menon, A.B. Pereira, G.M. Kirsztajn, et al., Influence of muscle mass and physical activity on serum and urinary creatinine and serum Cystatin C, Clin. J. Am. Soc. Nephrol. CJASN. 3 (2) (2008 Mar) 348–354.
[27] H. Li, X. Yuan, L. Liu, J. Zhou, C. Li, P. Yang, et al., Clinical evaluation of various thyroid hormones on thyroid function, Int. J. Endocrinol. 2014 (2014) 618572.