Title

Effects of Acute Kidney Injury and Chronic Hypoxemia on Fibroblast Growth Factor 23 Levels in Pediatric Cardiac Surgery Patients

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Effects of Acute Kidney Injury and Chronic Hypoxemia on Fibroblast Growth Factor 23 Levels in Pediatric Cardiac Surgery Patients

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by

Mark Robert Hanudel

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ABSTRACT OF THE THESIS

Effects of Acute Kidney Injury and Chronic Hypoxemia on Fibroblast Growth Factor 23 Levels in Pediatric Cardiac Surgery Patients

by

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Master of Science in Clinical Research

University of California, Los Angeles, 2016

Professor Robert M. Elashoff, Chair

Background:

Fibroblast growth factor 23 (FGF23) levels are elevated in cardiopulmonary bypass (CPB)-associated acute kidney injury (AKI); however, it is unknown how much of the circulating FGF23 is intact and bioactive. Hypoxia may induce FGF23 production, yet its impact in humans is unknown. Pediatric cardiac surgery patients have both a high incidence of CPB-associated AKI and a high prevalence of chronic hypoxemia.

Methods:

We assessed the effects of hypoxemia and CPB-associated AKI on C-terminal FGF23 (cFGF23) and intact FGF23 (iFGF23) levels in 32 pediatric cardiac surgery patients with normal eGFR. Plasma cFGF23 and iFGF23 were measured pre-operatively and serially post-operatively.

Results:

Despite normal renal and ventricular function, pre-operative cFGF23 levels were high and elevated out of proportion to iFGF23 levels. Pre-operative oxygen saturation correlated inversely with FGF23. Pre-operative cFGF23 and oxygen saturation both predicted post-operative AKI. Post-operatively, cFGF23 and iFGF23 increased early post-reperfusion; although cFGF23 levels remained elevated, iFGF23 levels soon returned to baseline.

Conclusions:

Pre-operative cFGF23 may predict CPB-associated kidney dysfunction. Changes over time in cFGF23 and iFGF23 levels post-CPB differ. Chronic hypoxemia may affect FGF23 production in humans.

The thesis of Mark Robert Hanudel is approved.

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2016

Table of Contents

List of Figures and Tables:

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Chapter 1: Manuscript

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Introduction:

Fibroblast growth factor 23 (FGF23) is secreted by osteocytes; it induces phosphaturia and decreases renal 1α-hydroxylase expression (1), physiologically functioning as a homeostatic regulator of phosphate and as a calcitriol counterregulatory hormone. In the setting of chronic kidney disease (CKD), circulating FGF23 levels increase as renal function declines (2,3) and are associated with progressive kidney disease (4,5) and increased cardiovascular morbidity and mortality (5,6).

Although FGF23 has been studied extensively in CKD, much less is known about FGF23 in the context of acute kidney injury (AKI). In a murine model of AKI, circulating FGF23 levels increased as early as one hour after AKI induction, and did so independently of phosphate, calcitriol, and PTH (7). Studies in humans with AKI have demonstrated that FGF23 concentrations, determined by the C-terminal assay, are increased in both adult (7-9) and pediatric (10) patients, and that these elevated levels are associated with poor clinical outcomes (8,9). However, it is unknown whether the high FGF23 levels in AKI reflect the intact, bioactive molecule or C-terminal fragments.

Not only are FGF23 levels increased in the setting of AKI, but in a small case-control study of pediatric cardiac surgery patients, Ali et al demonstrated that those patients who developed post-operative AKI had higher pre-operative FGF23 levels (10). The

etiology of these elevated pre-operative levels is unclear, but subclinical kidney dysfunction (10) or poor cardiac function (11) may have been contributory. Yet, other factors may also promote FGF23 production. Hypoxia has been shown to induce FGF23 expression in osteoblast-like cell lines (12,13) and increase circulating FGF23 levels in rats (13); however, its impact in humans is unknown.

The current study aims to: (i) determine whether or not chronic hypoxemia is associated with FGF23 levels in humans; (ii) assess the ability of pre-operative FGF23 levels to predict post-operative AKI; and (iii) to determine how measurements of C-terminal and intact FGF23 are affected by AKI. The study population is comprised of pediatric patients undergoing cardiopulmonary bypass (CPB), which is a population well-suited for our study, as cyanotic (hypoxemic) congenital heart disease is not uncommon in the pediatric cardiology population (14), and there is a high incidence of CPB-associated AKI after surgical correction (15).

Methods:

Study participants

Study participants were recruited from the UCLA Pediatric Cardiothoracic Surgery program. Patients aged 0 to 18 years scheduled to undergo cardiac surgery requiring CPB were eligible. An additional inclusion criterion was an estimated glomerular filtration rate (eGFR) of ≥60 ml/min/1.73m^2 at the time of surgery. Outpatient subjects were enrolled at their pre-operative clinic visits, which for almost all subjects occurred

within six days of their operative dates, and inpatient subjects were enrolled from the wards within two days prior to surgery. The study was approved by the UCLA Institutional Review Board, and informed consent and assent were obtained from parents and patients accordingly.

Following inclusion, demographic data were obtained from the medical records. Pre-operative oxygen saturation measurements via pulse oximetry were recorded from the pre-operative clinic visit note, the pre-operative anesthesia note, or the pre-operative inpatient note. In order to verify stable oxygen saturations over time, we also recorded oxygen saturations documented at least one week, and preferably one month, pre-operatively, and compared these remote measurements to the pre-operative values.

Study procedures

Plasma and serum were collected pre-operatively and at 2, 6, 12, 24, and 48 hours post-reperfusion. For outpatients, pre-operative blood was collected on the day of the pre-operative clinic visit, which occurred at a median of 3 (1, 4) days prior to surgery. For inpatients, pre-operative blood was collected 1-2 days prior to the operative date. Samples were placed on ice, centrifuged, aliquoted, and stored at -80^{0}C. Pre-operative and post-operative plasma C-terminal and intact FGF23 were measured in duplicate using standard ELISA kits (Immutopics, San Clemente, CA). Whereas the C-terminal assay detects both C-terminal FGF23 fragments and the intact form of the hormone, thus functioning as a surrogate measure of overall FGF23 production, the intact assay detects only the bioactive form of the hormone. Pre-operative and post-operative serum

parathyroid hormone (PTH) was assayed using a second-generation assay (Immutopics). Pre-operative plasma B-type natriuretic peptide (BNP) was measured using the Triage® Kit (Alere, Waltham, MA).

As part of routine pre-operative care, serum creatinine, phosphate, and calcium were measured prior to surgery. Post-operatively, in the Cardiothoracic Intensive Care Unit, serum creatinine, phosphate, and calcium were measured daily. AKI was defined as an increase in serum creatinine of at least 50% from pre-operative levels, as established by the Acute Kidney Injury Network (16), occurring within 48 hours of surgery. A large study of pediatric cardiac surgery patients demonstrated that most patients who develop AKI, defined as a 50% increase in creatinine, did so within 24 hours of surgery, and almost all did so within 48 hours of surgery (15). As our serum creatinine measurements were determined by the alkaline picrate method (Jaffe method), eGFR was calculated by the original Schwartz equation (17,18). All pre-operative echocardiograms were interpreted by the same pediatric cardiologist.

Statistical analysis

Data are reported as means and standard errors of the means (SEM), or medians and interquartile ranges (IQR), as was suitable for the data distributions. Continuous or ordinal pre-operative variables were compared between groups using the Student t-test or the Mann-Whitney U-test, according to the data distributions. Categorical variables between groups were compared using the Fisher exact test. Comparisons were two-tailed, with $p<0.05$ considered significant. Correlations between pre-operative oxygen

saturations and FGF23 levels were analyzed using Pearson product-moment correlation coefficients.

C-terminal FGF23 (cFGF23) levels vary physiologically with age in the pediatric population, decreasing from a median value of 105 (75, 153) RU/ml in infants to median values in the 60-80 RU/ml range for children and adolescents (19). To adjust for age, we calculated pre-operative age-related cFGF23 standard deviation scores (SDS), utilizing cFGF23 data obtained from a large, age-specific cohort of healthy children, determined by the Immutopics assay, as described by Fischer et al (19). The SDS, also known as a z-score, is defined as the number of standard deviations an observation is above the mean. Phosphate levels also vary with age, and published data was used to calculate pre-operative age-adjusted phosphate SDS (20). As age-adjusted normal values do not exist for intact FGF23 (iFGF23), the absolute value of this parameter was used in all analyses. For comparison, we also measured iFGF23 and cFGF23 levels, using the same Immutopics assays, in an institutional cohort of 47 healthy children.

Potential pre-operative predictors of post-operative kidney dysfunction were assessed for two outcomes: AKI vs. no AKI (dichotomous outcome), and maximum percent decrease in eGFR (continuous outcome). Receiver operating characteristic (ROC) curve analysis and multiple logistic regression modeling were used to assess the ability of pre-operative factors to predict post-operative CPB-associated AKI. Multiple linear regression modeling was used to assess the ability of pre-operative factors to predict post-operative percent decrease in eGFR. Regression models included FGF23 values

or oxygen saturation, age, and CPB time; age and CPB time are known risk factors for CPB-associated AKI (15).

Linear mixed effects modeling was utilized to examine changes in biochemical parameters over time. Given the data distributions, both cFGF23 and iFGF23 values were log-transformed prior to modeling. The percent change over time in untransformed cFGF23 and iFGF23 levels was also calculated.

All statistical analysis was performed using SigmaPlot 11.0 (San Jose, CA) software, with the exception of linear mixed effects modeling, which was performed using SAS (Cary, NC) software.

Results:

Pre-operative patient characteristics

Table 1 shows pre-operative characteristics of the 32 study patients, including the overall cohort and by post-operative status (AKI vs. non-AKI). The median age was 37 (10, 80) months, and mean eGFR was 141 ± 42 ml/min/1.73m^2. cFGF23 levels were elevated compared to controls (295 [109, 547] vs. 59 [39, 76] RU/ml, p<0.001), as was the mean age-adjusted cFGF23 standard deviation score (SDS) (2.28 ± 0.34 vs. -0.59 ± 1.50, p<0.001). iFGF23 levels, however, were similar to controls (56 [38, 72] vs. 50 [35, 87] pg/ml, p=0.84). Importantly, whereas cFGF23 values tended to negatively correlate with age, age-adjusted cFGF23 SDS did not (**Figure 1**).

Ventricular function, assessed qualitatively by echocardiogram, was normal in 29 patients (91%); 3 patients had mildly depressed function. The surgical procedures performed are listed in **Table 2**. Post-operatively, 20 patients (62.5%) developed at least Stage 1 AKI by the Acute Kidney Injury Network (AKIN) criteria (16). The AKI group was younger than the non-AKI group, and thus had lower pre-operative creatinine; however, pre-operative eGFR did not differ between the groups. Pre-operative cFGF23 levels, as well as age-adjusted cFGF23 SDS, were higher in the AKI group than in the non-AKI group; however, iFGF23 levels did not differ between the groups. Pre-operative oxygen saturation (SpO_2) was lower in the AKI group (**Table 1**). Of the 15 patients with pre-operative SpO_2 <95%, cardiac surgery improved saturations in all patients, and normalized saturations in 10 patients.

Post-operative renal function

Peak post-operative percent change in serum creatinine was 74 (67, 100) % in the AKI group and 21 (0, 25) % in the non-AKI group ($p<0.001$). Of the AKI patients, all developed AKI within 24 hours of surgery, with the exceptions of two patients who were diagnosed with AKI at 26 hours and 39 hours post-reperfusion. A large prospective study of pediatric cardiac surgery patients observed a similar early development of CPB-associated AKI (15). Percent change in creatinine over time is shown in **Figure 2a**. Serum creatinine levels returned to baseline by post-operative day 5 in all patients. No patient became oliguric post-operatively or required renal replacement therapy.

Post-operative changes in cFGF23 levels

For all patients, the median peak cFGF23 level was 1629 (596, 5085) RU/ml, with a median 6 (2, 18) fold change. cFGF23 levels increased early and markedly post-reperfusion (**Figure 2b**). Linear mixed effects modeling revealed that log-transformed cFGF23 levels differed over time. Using the Benjamini and Hochberg correction for multiple comparisons, log-transformed cFGF23 levels were higher than pre-operative values at 2 hours post-reperfusion ($p<0.001$), 6 hours post-reperfusion ($p=0.002$), 12 hours post-reperfusion ($p=0.03$), 24 hours post-reperfusion ($p<0.001$), and 48 hours post-reperfusion ($p=0.02$). Group status (AKI vs. non-AKI) did not modify the effect of time on log-transformed cFGF23 levels. The percent increase from baseline in untransformed cFGF23 levels was large, sustained over time, and did not differ between groups (**Figure 3**).

Post-operative changes in iFGF23 levels

For all patients, the median peak iFGF23 level was 84 (62, 152) pg/ml, with a median 1.7 (1.2, 2.6) fold change. Like cFGF23 levels, iFGF23 levels increased in the early post-operative period; however, unlike cFGF23 levels, iFGF23 levels returned to baseline soon thereafter (**Figure 2c**). Log-transformed iFGF23 levels were higher than pre-operative values at 2 hours post-reperfusion ($p=0.001$); did not differ from pre-operative values at 6 hours post-reperfusion ($p=0.38$), 12 hours post-reperfusion ($p=0.31$), and 24 hours post-reperfusion ($p=0.52$); and were lower than pre-operative values at 48 hours post-reperfusion ($p=0.01$). The percent increase from baseline in untransformed iFGF23 levels was not nearly as dramatic as that of cFGF23 levels, and

was only observed through 24 hours post-reperfusion (**Figure 3**). Although percent change from baseline in untransformed iFGF23 levels seemed notably higher in the AKI group at 2 hours post-reperfusion (75 ± 72 vs. 36 ± 40 %) and at 12 hours post-reperfusion (51 ± 198 vs. -32 ± 25 %), these differences did not reach statistical significance.

Post-operative changes in phosphate, calcium, and PTH levels

Post-operative serum phosphate concentrations tended to be higher than pre-operative values at 13 hours post-reperfusion ($p=0.05$), but were lower than pre-operative values at 37 hours post-reperfusion ($p<0.001$) and 61 hours post-reperfusion ($p<0.001$) (**Figure 2d**). At their peak, serum phosphate levels increased by 21 ± 8 % in the AKI group and 2 ± 9 % in the non-AKI group ($p=0.12$). Post-operative serum calcium concentrations were lower than pre-operative values at 13, 37, and 61 hours post-reperfusion ($p<0.001$ for all) (**Figure 2e**). The nadir decrease in serum calcium levels was 10 ± 2 % in the AKI group and 7 ± 2 % in the non-AKI group ($p=0.21$). PTH levels increased over time to a greater degree in the AKI group than in the non-AKI group ($p=0.04$, **Figure 2f**).

Pre-operative FGF23 as a predictor of CPB-associated AKI

Pre-operative cFGF23 SDS predicted post-operative CPB-associated AKI (**Figure 4**). No single predictor variable (age, oxygen saturation, or cFGF23 SDS) predicted AKI significantly better than the others. Combinations of the predictor variables also predicted AKI (**Table 3**); however, after correcting for multiple comparisons, no combination of variables predicted AKI significantly better than any variable alone. In

multiple logistic regression modeling, after adjusting for age and CPB time, pre-operative cFGF23 SDS was positively associated with AKI (OR = 2.07 (95% CI: 1.13, 3.79), p=0.02) (**Table 4**). Yet, given the loss of power inherent to outcome dichotomization (AKI vs. no AKI), and given that decrements in kidney function of less than 50% (not meeting the AKI threshold) may still be relevant, we also assessed a continuous outcome variable, the percent decrease in eGFR. Multiple linear regression modeling revealed that, after adjusting for age and CPB time, pre-operative cFGF23 SDS was positively associated with post-operative percent decrease in eGFR (B = 3.48 (95% CI: 0.97, 5.98), p=0.008) (**Table 5**). All three covariates were significantly associated with the outcome. The addition of cFGF23 SDS as a covariate to a model that already included age and CPB time increased the adjusted R^2 from 0.46 to 0.57, indicating a relatively large increase in the degree to which the covariates explain the variability in the outcome measure. Using cFGF23 SDS as a covariate, as opposed to cFGF23 levels, allowed us to assess the age-independent effects of cFGF23 and avoid the collinearity that arose when including cFGF23 levels with age in the model. After adjustment for age and CPB time, iFGF23 levels were not associated with post-operative percent decrease in eGFR (p=0.33) (**Table 6**).

Association of pre-operative oxygen saturation and FGF23 levels

Pre-operative oxygen saturation (SpO_2) inversely correlated with cFGF23 SDS (r = -0.65, p<0.001), cFGF23 levels (r = -0.48, p=0.005), and iFGF23 levels (r = -0.35, p=0.047) (**Figure 5**). Previous SpO_2 values measured at a median of 37 (24, 54) days prior to the pre-operative SpO_2 determinations were highly correlated with the pre-

operative measurements (r = 0.96, p<0.001), suggesting stability of the oxygen saturation measurements over time. In multiple linear regression modeling, pre-operative SpO_2 remained negatively associated with cFGF23 SDS, independent of serum phosphate (SpO_2 p=0.002) (**Table 7**) or phosphate SDS (SpO_2 p<0.001) (**Table 8**).

As chronic hypoxemia may adversely affect kidney function (22-24), potentially rendering the kidney less tolerant of CPB, we hypothesized that pre-operative SpO_2 may also predict post-operative CPB-associated AKI. Like cFGF23 SDS, pre-operative SpO_2 predicted post-operative CPB-associated AKI (**Figure 4**). In regression analyses, given its high correlation with cFGF23 SDS, pre-operative SpO_2 was not included as a predictor covariate in our original models. However, after excluding cFGF23 SDS from the models, and adjusting for age and CPB time, pre-operative SpO_2 was negatively associated with AKI in logistic regression (OR = 0.83 (95% CI: 0.71, 0.98), p=0.03) (**Table 9**), and negatively associated with post-operative percent decrease in eGFR in linear regression (B = -0.47 (95% CI: -0.91, -0.02), p=0.04) (**Table 10**). The addition of SpO_2 as a covariate to a linear regression model that already included age and CPB time increased the adjusted R^2 from 0.46 to 0.52.

Discussion:

The current results confirm previous observations demonstrating elevated cFGF23 levels in AKI patients (7-10), as well as the ability of cFGF23 to predict the development

of AKI (10), as defined by an increase in serum creatinine of at least 50% from baseline. However, we also present the novel finding that, in patients with cardiopulmonary bypass-associated acute kidney dysfunction, cFGF23 levels remain elevated for a longer duration than iFGF23 levels. Furthermore, in pediatric congenital heart disease patients, we show that chronic hypoxemia is associated with elevated FGF23 levels.

In the setting of acute kidney dysfunction, there is an early and marked elevation in cFGF23 levels, as previously described in a murine AKI model (7). C-terminal levels increased considerably in our AKI group, and even increased 5-fold in the non-AKI group, in whom there was only a 21% median peak increase in creatinine levels. The increase of cFGF23 levels in the non-AKI group may be attributable to subclinical kidney dysfunction or to an inflammatory response. The observation that cFGF23 levels increased out of proportion to the degree of acute kidney dysfunction has been previously observed and ascribed, at least in part, to a systemic inflammatory response to surgery (7). Indeed, in CKD patients, higher cFGF23 levels are independently associated with elevated markers of inflammation (25). Proposed mechanisms by which circulating FGF23 levels may increase in AKI include increased production from bone; increased ectopic production, possibly from damaged renal tubules; decreased renal clearance; decreased degradation; and/or release of stored, preformed hormone (7,26). Although hyperphosphatemia may represent a stimulus for FGF23, circulating FGF23 levels increased before phosphate concentrations peaked. Furthermore, in the non-AKI group, FGF23 levels increased by 2 hours post-reperfusion despite a slight decrease in serum phosphate.

Although circulating FGF23 is nearly all intact and biologically active in patients with end-stage kidney disease (27), the correlation between measurements made by C-terminal and intact FGF23 assays is not as strong in individuals with preserved kidney function (28). We measured FGF23 levels using both the C-terminal and intact assays; whereas the C-terminal assay measures total FGF23 levels, the intact assay detects only the bioactive form of the hormone. Our study sample had a median pre-operative cFGF23 level of 295 RU/ml (range 59-2653 RU/ml) and a median iFGF23 level of 56 pg/ml (range 27-129 pg/ml). As cFGF23 levels vary with age, we used pre-operative age-adjusted cFGF23 standard deviation scores in our analyses, calculated as detailed by Fischer et al (19). Although the cFGF23 levels were clearly elevated for age (mean age-adjusted SDS of 2.28) (19), similar age-adjusted normal values do not exist for iFGF23. However, the iFGF23 values were similar to values observed in our institutional cohort of 47 healthy children (mean age 11.3 years). Although this cohort is older than our study sample, it is unlikely that normal iFGF23 levels in younger children would be so much lower as to suggest that our observed pre-operative iFGF23 values were dramatically elevated. Indeed, in our healthy cohort, iFGF23 tended to correlate negatively with age ($r = -0.28$, $p=0.06$); similarly, cFGF23 levels tend to be higher, not lower, in younger children (19). Therefore, the study sample had unexpectedly high pre-operative cFGF23 levels that were elevated out of proportion to iFGF23 levels. These observations suggest that, although pre-operative FGF23 production may have been increased, proteolytic cleavage mechanisms (29) may have been concurrently

upregulated, maintaining levels of intact FGF23 that are closer to the normal range, similar to what is thought to occur in the setting of iron deficiency (30-32).

Post-operatively, both cFGF23 and iFGF23 levels increased by 2 hours post-reperfusion; however, cFGF23 remained elevated for a longer duration than iFGF23 levels. This discrepancy suggests that different factors may affect FGF23 transcription and post-translational cleavage. Group status (AKI vs. non-AKI) did not modify the effect of time on cFGF23 levels, suggesting that overall FGF23 translation, of which cFGF23 levels may be considered a surrogate measure, was affected less by decreased GFR and more by other factors such as inflammation. Moreover, the generally similar trends in FGF23 levels over time between the AKI and non-AKI groups suggest that factors besides kidney function affected circulating FGF23 concentrations.

Interestingly, pre-operative serum phosphate concentrations were relatively low for age in the study sample (mean age-adjusted phosphate SDS of -1.49). Given that pre-operative iFGF23 values were similar to what we observed in the cohort of healthy children, it seems unlikely that iFGF23 was causing the relative hypophosphatemia. The effects of PTH and 1,25$(OH)_2$ vitamin D may have played a role. However, relative malnutrition may have been more contributory, as evidenced by the cohort's below-average growth parameters (mean age-adjusted weight SDS of -1.49, and mean age-adjusted height SDS of -1.22).

Post-operatively, phosphate values decreased to below pre-operative levels by 37 hours post-reperfusion. It is unlikely that this post-operative relative hypophosphatemia was entirely caused by elevated iFGF23 levels, which peaked at 2 hours post-reperfusion. Nutritional status may have been contributory, as many patients in the early post-operative period remained on intravenous fluids and had yet to be advanced to full enteral feeds or parenteral nutrition. It is possible that the elevated PTH levels, at least in the AKI group, also contributed to the hypophosphatemia. Decreased $1,25(OH)_2$ vitamin D levels may also have contributed, although in the setting of nearly recovered renal function, lower iFGF23 levels and higher PTH levels would be expected to increase $1,25(OH)_2$ vitamin D production. However, the relative hypophosphatemia may have contributed to the decrease in iFGF23 levels to below baseline at 48 hours post-reperfusion.

Similar to a previous study of FGF23 levels in the pediatric CPB population (10), we observed that pre-operative cFGF23 levels and cFGF23 SDS were markedly higher in those patients who went on to develop AKI. It is unclear why higher pre-operative FGF23 levels are associated with worse post-operative renal function. It is unknown whether higher FGF23 levels have some direct pathologic effect promoting renal injury, or if FGF23 represents a surrogate marker for another factor that is associated with worse post-operative renal function. It has been hypothesized that subclinical kidney dysfunction may contribute to both high pre-operative FGF23 levels and post-operative CPB-associated AKI (10). Cardiac status may also be contributory. In a study of pediatric heart failure patients with normal mean eGFR, FGF23 levels were elevated

compared to controls and correlated with heart failure severity (11). As such, high pre-operative FGF23 levels and post-operative kidney dysfunction may, in part, be attributable to poor cardiac function. However, our study sample did not have heart failure, as evidenced by low serum BNP values, which is a sensitive marker of heart failure (33,34), and qualitatively normal ventricular function on echocardiogram.

An interesting observation from the present study is the potential role of hypoxemia in the association between high pre-operative cFGF23 levels and post-operative kidney dysfunction. Pre-operative cFGF23 levels and oxygen saturation negatively correlated, and both variables predicted kidney dysfunction, suggesting that elevated cFGF23 levels possibly represented a surrogate marker for hypoxemia. Chronic hypoxia affects renal cellular function (22-24), and small studies of pediatric patients with cyanotic congenital heart disease have demonstrated evidence of tubular and glomerular damage (35-37). As such, some degree of cyanotic nephropathy-associated renal compromise may have rendered the kidneys of our hypoxemic patients less tolerant of CPB and possibly more susceptible to AKI.

The associations between hypoxemia and FGF23 levels in humans have not been previously described; however, increased FGF23 expression has been observed in osteoblast-like cell lines under hypoxic conditions (12,13), and elevated circulating levels have been observed in hypoxic rats (13). Compared to controls, the hypoxic rats had much higher cFGF23 levels, but comparable iFGF23 levels (13). In the current

study, SpO_2 negatively correlated with FGF23 levels, suggesting an association between chronic hypoxemia and increased FGF23 production.

Limitations of our study include the small sample size, the relatively short duration of post-operative measurement, incomplete biochemical assessment (including lack of $1,25(OH)_2$ vitamin D levels and inflammatory markers), and the observational nature of our findings. Furthermore, this study sample developed only mild AKI; therefore, the changes in iFGF23 may be underestimated given the lesser degree of renal dysfunction.

In summary, although elevated FGF23 levels have been previously described in CPB-associated AKI, the current findings provide new insights into how AKI may affect FGF23 levels. In mild AKI, cFGF23 levels are increased for a longer duration than iFGF23 levels, possibly suggesting alterations in cleavage mechanisms. Pre-operatively, age-adjusted cFGF23 SDS may predict CPB-associated kidney dysfunction. Furthermore, for the first time in humans, we have identified an association between chronic hypoxemia and FGF23 levels. Further studies will be needed to elucidate the mechanisms by which hypoxemia and acute kidney dysfunction may result in elevated FGF23 levels.

Table 1: Pre-operative cohort characteristics: overall and by post-operative AKI status

	All	AKI Patients	Non-AKI Patients	p value (AKI vs. Non-AKI)	Controls	p value (All vs. Controls)
Number	32	20	12	n/a	47	n/a
Sex (% male)	56	60	50	0.72	--	--
Age (months)	37 (10, 80)	15 (7, 49)	91 (41, 146)	***0.005***	132 (108, 180)	***<0.001***
Weight SDS	-1.49 ± 0.33	-1.36 ± 0.35	-1.70 ± 0.67	0.63	--	--
Height SDS	-1.22 ± 0.28	-1.22 ± 0.36	-1.22 ± 0.45	1.00	--	--
Oxygen Saturation (%)	96 (80, 99)	87 (74, 98)	99 (96, 100)	***0.01***	--	--
Hemoglobin (g/dl)	13.1 (12.1, 16.3)	15.0 (12.5, 17.3)	12.7 (11.9, 13.2)	0.07	--	--
BNP (pg/ml)	<20 (<20,<20)	<20 (<20,<20)	22.5 (<20, 74)	n/a	--	--
RACHS-1 Score (21)	2 (2, 3)	2 (2, 3)	2 (2, 2)	0.07	--	--
CPB Time (min)	44 (34, 68)	46 (35, 65)	42 (33, 76)	0.61	--	--
AXC Time (min)	21 (16, 45)	22 (9, 41)	21 (17, 51)	0.98	--	--
Diuretic Use (%)	25	30	17	0.68	--	--
ACEI Use (%)	31	35	25	0.70	--	--
Creatinine (mg/dl)	0.38 ± 0.03	0.32 ± 0.03	0.48 ± 0.05	***0.003***	--	--
eGFR (ml/min/1.73m^2)	141 ± 42	144 ± 47	136 ± 33	0.65	--	--
Calcium (mg/dl)	10.1 ± 0.1	10.1 ± 0.1	9.9 ± 0.2	0.19	--	--
Phosphate (mg/dl)	4.9 ± 0.1	5.1 ± 0.2	4.4 ± 0.2	***0.01***	--	--
Phosphate SDS	-1.49 ± 0.27	-1.58 ± 0.34	-1.33 ± 0.46	0.66	--	--
PTH (pg/ml)	64 ± 5	60 ± 6	69 ± 10	0.39	--	--
C-terminal FGF23 (RU/ml)	295 (109, 547)	437 (232, 705)	119 (80, 153)	***0.006***	59 (39, 76)	***<0.001***
C-terminal FGF23 SDS	2.28 ± 0.34	2.81 ± 0.44	1.38 ± 0.42	***0.04***	-0.59 ± 1.50	***<0.001***
Intact FGF23 (pg/ml)	56 (38, 72)	57 (48, 76)	41 (36, 62)	0.19	50 (35, 87)	0.84

Table 2: Surgical procedures performed

Surgical Procedure	n
Tetralogy of Fallot repair	9
Ventricular septal defect repair	6
Fontan procedure	5
Mitral valve replacement	2
Pulmonary valve replacement	2
Subaortic stenosis resection	2
Arterial switch	1
Complete atrioventricular canal repair	1
Ebstein anomaly repair	1
Glenn procedure	1
Partial anomalous pulmonary venous return	1
Septal myomectomy	1

Figure 1: C-terminal FGF23 vs. age

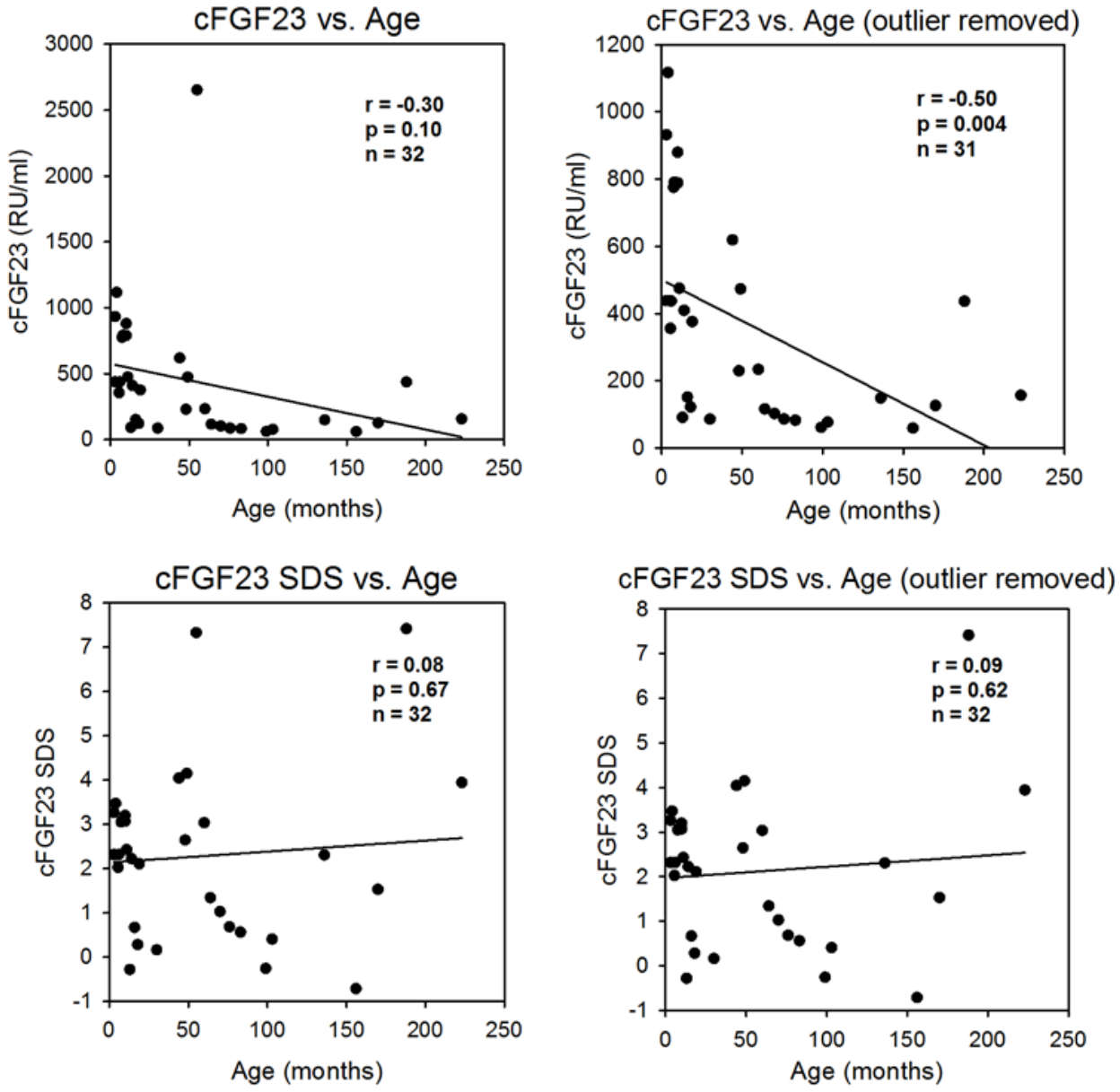


Figure 2: Biochemical parameters over time

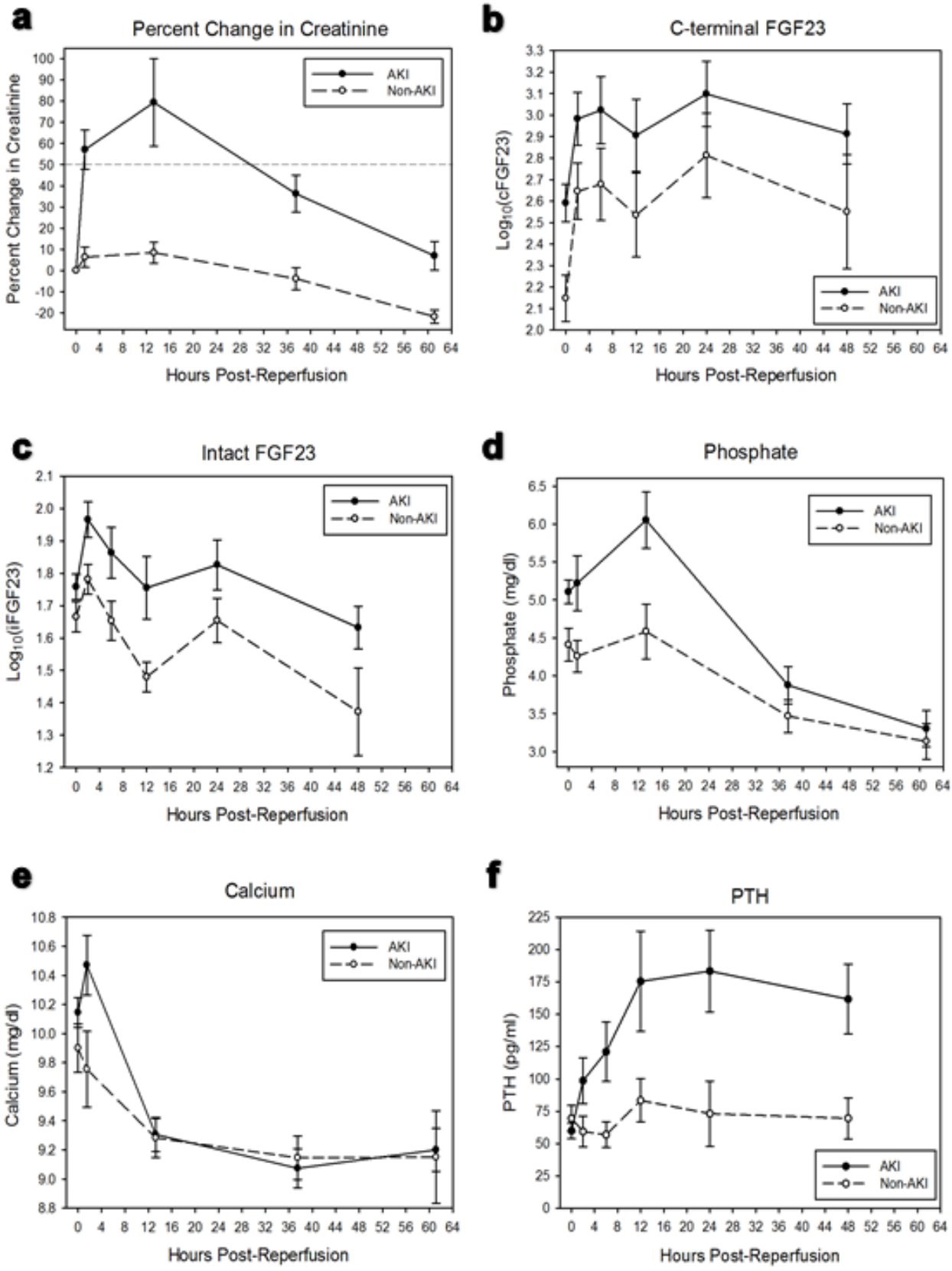


Figure 3: Percent change in FGF23 levels over time

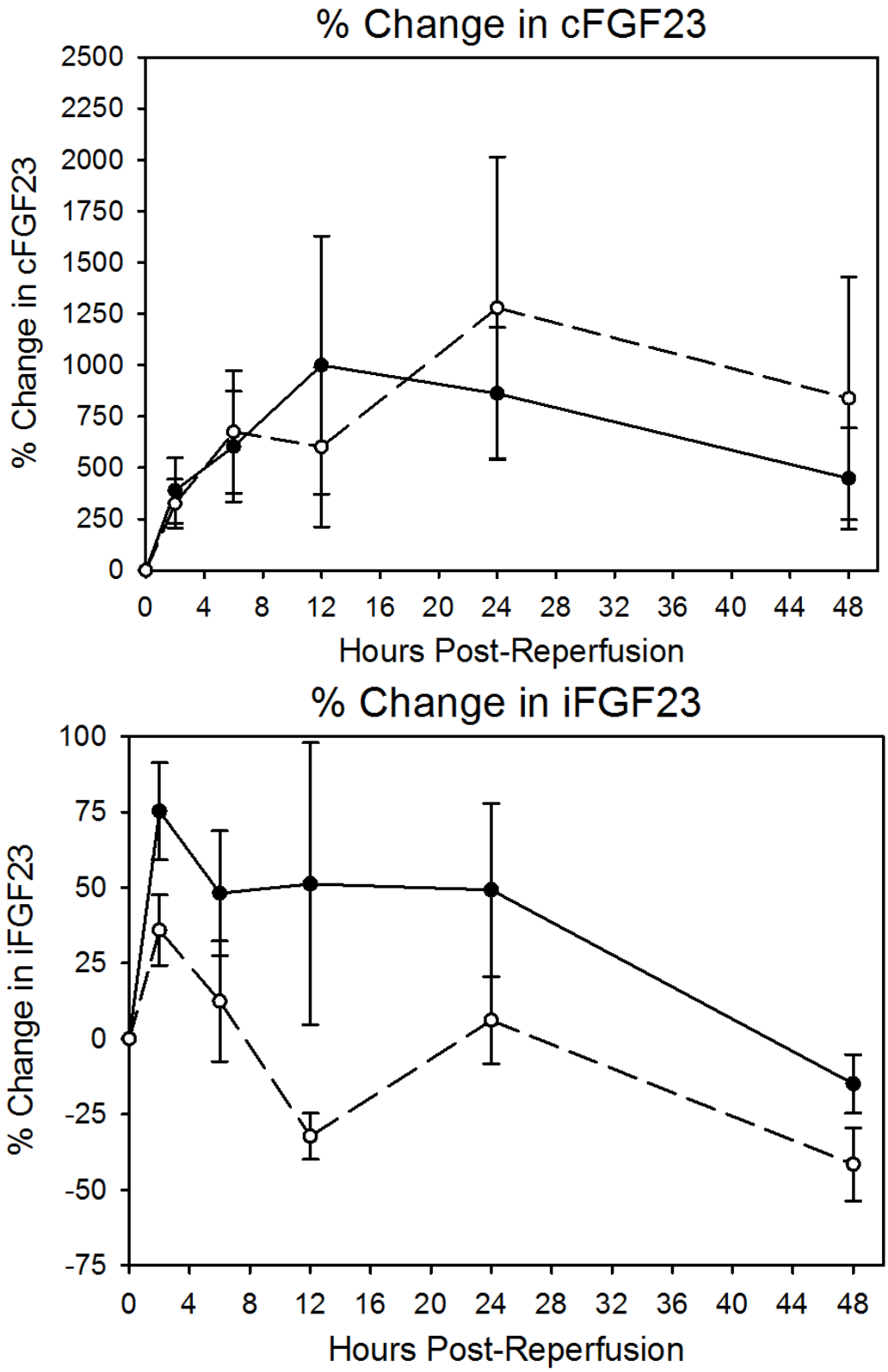


Figure 4: Receiver operating characteristic (ROC) curves for AKI prediction

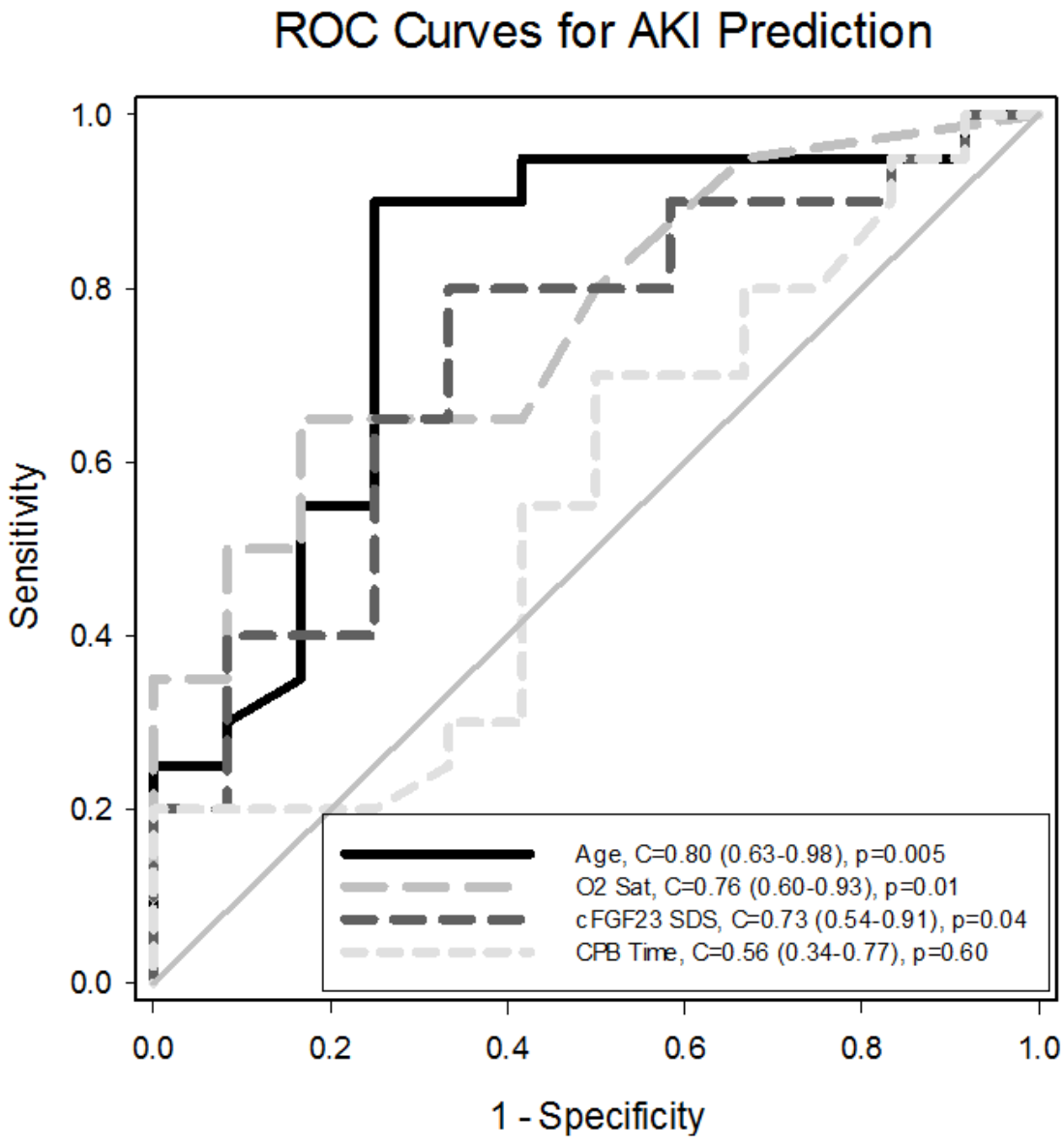


Table 3: Predictor variable combinations for AKI prediction

Predictor Variables	C-statistic	p-value
Age + Oxygen Saturation	0.91 (0.81, 1.00)	<0.001
Age + cFGF23 SDS	0.85 (0.69, 1.00)	<0.001
Oxygen Saturation + cFGF23 SDS	0.75 (0.58, 0.92)	0.02
Age + O2 Saturation + cFGF23 SDS	0.89 (0.76, 1.00)	<0.001

Table 4: Multiple logistic regression modeling (dependent variable: AKI or no AKI)

Independent Variable	Odds Ratio	p-value
Age (months)	0.74 (0.59, 0.93)	0.009
Cardiopulmonary bypass time (min)	1.01 (0.98, 1.04)	0.55
Pre-operative cFGF23 SDS	2.07 (1.13, 3.79)	0.02

Table 5: Multiple linear regression modeling (dependent variable: percent decrease in eGFR)

Independent Variable	B (95% CI)	p-value
Age (months)	-0.18 (-0.26, -0.10)	<0.001
Cardiopulmonary bypass time (min)	0.26 (0.11, 0.42)	0.001
Pre-operative cFGF23 SDS	3.48 (0.97, 5.98)	0.008

Table 6: Multiple linear regression modeling (dependent variable: percent decrease in eGFR)

Independent Variable	B (95% CI)	p-value
Age (months)	-0.16 (-0.25, -0.07)	0.001
Cardiopulmonary bypass time (min)	0.24 (0.07, 0.40)	0.001
Pre-operative iFGF23 (pg/ml)	0.11 (-0.12, 0.34)	0.33

Figure 5: Correlations between pre-operative FGF23 and oxygen saturation

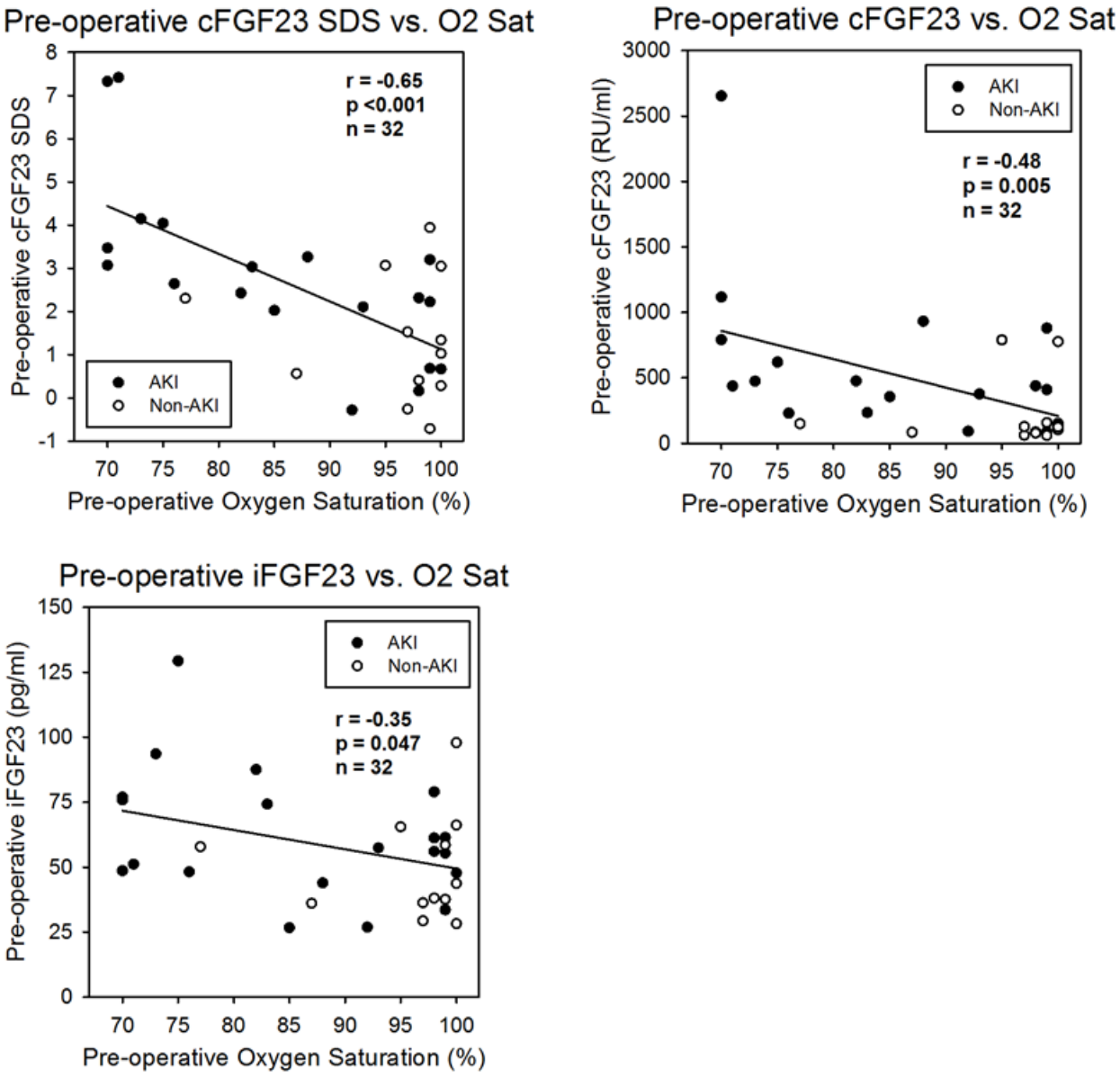


Table 7: Multiple linear regression modeling (dependent variable: cFGF23 SDS)

Independent Variable	B (95% CI)	p-value
Oxygen saturation (%)	-0.09 (-0.14, -0.04)	0.002
Phosphate (mg/dl)	0.24 (-0.51, 0.99)	0.51

Table 8: Multiple linear regression modeling (dependent variable: cFGF23 SDS)

Independent Variable	B (95% CI)	p-value
Oxygen saturation (%)	-0.10 (-0.15, -0.04)	<0.001
Phosphate SDS	0.01 (-0.37, 0.39)	0.97

Table 9: Multiple logistic regression modeling (dependent variable: AKI or no AKI)

Independent Variable	Odds Ratio	p-value
Age (months)	0.97 (0.94, 0.99)	0.02
Cardiopulmonary bypass time (min)	1.00 (0.97, 1.03)	0.96
Pre-operative SpO_2 (%)	0.83 (0.71, 0.98)	0.03

Table 10: Multiple linear regression modeling (dependent variable: percent decrease in eGFR)

Independent Variable	B (95% CI)	p-value
Age (months)	-0.17 (-0.25, -0.09)	<0.001
Cardiopulmonary bypass time (min)	0.23 (0.07, 0.39)	0.006
Pre-operative SpO_2 (%)	-0.47 (-0.91, -0.02)	0.04

Chapter 2: Statistical Appendix

To assess whether the assumptions of the multiple linear regression models are reasonable for the data set, we performed regression diagnostics. We evaluated the multiple linear regression model that included post-operative percent decrease in eGFR as the dependent variable, and age, CPB time, and pre-operative cFGF23 SDS as the independent variables (**Table 5**). We first plotted the observed values for the dependent variable vs. the predicted values for the dependent variable (**Figure 6**). For each data point, the difference between the observed and predicted values is the residual. To test the assumptions that the relationship between independent and dependent variables is linear, and that the residuals have equal variance, we plotted the residuals vs. the independent variables, the observed dependent variables, and the predicted dependent variables (38). As shown in **Figure 7**, the residuals seemed to be reasonably uniformly distributed in a symmetric band centered on a residual value of 0, suggesting that the model has been correctly specified and that the equal variance assumption is satisfied.

As the raw residuals are in the units of the dependent variable, it is difficult to define what constitutes an outlier. To better quantify the residual analysis, standardized residuals may be used, in which the raw residuals are divided by the standard deviation of the residuals (39). Standardized residuals are expected to be normally distributed. As shown in **Figure 8**, for the current model, most of the absolute values of the standardized residuals were less than 2. Two standardized residuals had absolute values above this threshold (2.07 and 2.17), representing ~6% of the data set of 32 points. However, as 5% of the standardized residuals should exceed 2 simply due to the

nature of the normal distribution, such values are expected and would not necessarily warrant consideration as outliers (39). No standardized residual had an absolute value greater than 3, which would more certainly identify a potential outlier.

We also used the standardized residuals to test the assumption that the residuals are normally distributed (40). Based on the reasonable linearity of the normal probability plot (**Figure 9**), the data seem consistent with the normality assumption. Furthermore, we formally tested the hypothesis that the residuals are normally distributed by testing whether the correlation coefficient between the residuals and the corresponding cumulative frequency values is significantly different from 0 (41). As r (0.96) significantly differed from 0 ($p<0.001$), we concluded that the residuals are indeed normally distributed.

Next, for each independent variable, we assessed the leverage of each point. A data point was identified as a high leverage point if the calculated leverage value was greater than twice the expected value of the leverage, defined as $(k+1)/n$, where k is the number of independent variables (model threshold = 0.25) (42). The following points were identified as high leverage points: For the independent variable age, x=223 months; for the independent variable CPB time, x=165 minutes; and for the independent variable cFGF23 SDS, x=7.33 and x=7.42. However, high leverage only represents the potential to influence the regression results, as the actual influence also depends on the observed value of y (43). Indeed, the standardized residuals associated with these four

high leverage points ranged from -0.28 to 0.93, suggesting little actual influence on the regression.

Standardized residuals are normalized by dividing by the residual standard deviation, which is constant for all values of X (44). However, given the effects of leverage, residual normalization may be improved by dividing the residuals by their specific standard errors, which is a function of X (45). The distribution of Studentized residuals (**Figure 10**) resembled that of the standardized residuals. As with the standardized residuals, most of the absolute values of the Studentized residuals were less than 2. Two Studentized residuals had absolute values above this threshold (2.21 and 2.25), although, again, identifying two such values in this data set would be expected given the nature of the normal distribution. No Studentized residual had an absolute value greater than 3.

Whereas Studentized residuals are calculated using all of the data, Studentized deleted residuals are computed after deleting the point associated with the residual (45). The distribution of Studentized deleted residuals (**Figure 10**) resembled that of the Studentized residuals and the standardized residuals. As with the standardized and Studentized residuals, most of the absolute values of the Studentized deleted residuals were less than 2. Two Studentized deleted residuals had absolute values above this threshold (2.39 and 2.44), as would be expected. No Studentized residual had an absolute value greater than 3.

To further assess for outliers that may exert undue influence on the regression results, we plotted the Studentized deleted residuals vs. leverage (**Figure 11**). No high leverage data point (leverage value greater than 0.25) had an absolute value for the associated Studentized deleted residual that was greater than 2. Conversely, no data point that had an absolute Studentized deleted residual value of greater than 2 was a high leverage point. As no data points had high values for both leverage and associated Studentized deleted residual (hashed areas in **Figure 11**), no data points seem to be extreme outliers or excessively influential points.

Whereas the regression diagnostics presented above function to identify data points that may excessively influence the regression analysis, another statistic, Cook's distance, directly assesses the actual influence of each data point on the regression equation by computing how much the regression coefficients change when the point is deleted (46). Some sources state that data points with Cook's distance values greater than 1 are considered to warrant further investigation, and data points with Cook's distance values greater than 4 are considered to be outliers (47). However, other sources state that the cutoff should be 4/n (48). Using the more conservative 4/n cutoff, our model threshold is 0.125. This diagnostic identified three data points, with Cook's distance values of 0.17, 0.19, and 0.23 (**Figure 12**), which may be somewhat influential.

Another influence diagnostic statistic is DFFITS ("difference in fit statistic"). For each data point, the DFFITS value is the number of estimated standard errors that the predicted value changes if that data point is removed from the data set (47). Absolute

DFFITS values greater than twice the square root of (k+1)/n (model threshold = 0.71) are considered to indicate an influential point (49). The DFFITS diagnostic identified the same three points, with absolute DFFITS values of 0.90, 0.90, and 0.97 (**Figure 12**), which may be somewhat influential.

These three potentially influential points were associated with a subject that had no decline in kidney function (data point #29), the subject that had the maximal decrease in kidney function (data point #18), and the second-oldest subject that also had the highest cFGF23 SDS (data point #11). **Figure 13** highlights these three data points in the other regression diagnostics. We repeated the multiple linear regression model, excluding these three subjects with relatively extreme data. All three independent variables remained significantly associated with the dependent variable, and the adjusted R^2 only decreased from 0.57 to 0.55.

Regarding the multiple linear regression model that included post-operative percent decrease in eGFR as the dependent variable, and age, CPB time, and oxygen saturation as the independent variables (**Table 10**), there seemed to be one particularly influential outlier (data point #29). This data point had the highest absolute residual (-32%), highest standardized residual (-2.37), highest Studentized residual (-2.60), highest Studentized deleted residual (-2.93), highest Cook's distance value (0.34), highest DFFITS value (-1.31), and the fifth highest leverage value (0.17). Excluding this potentially influential point resulted in a somewhat improved model, increasing the adjusted R^2 from 0.52 to 0.60.

Figure 6: Multiple linear regression observed vs. predicted percent decrease in eGFR

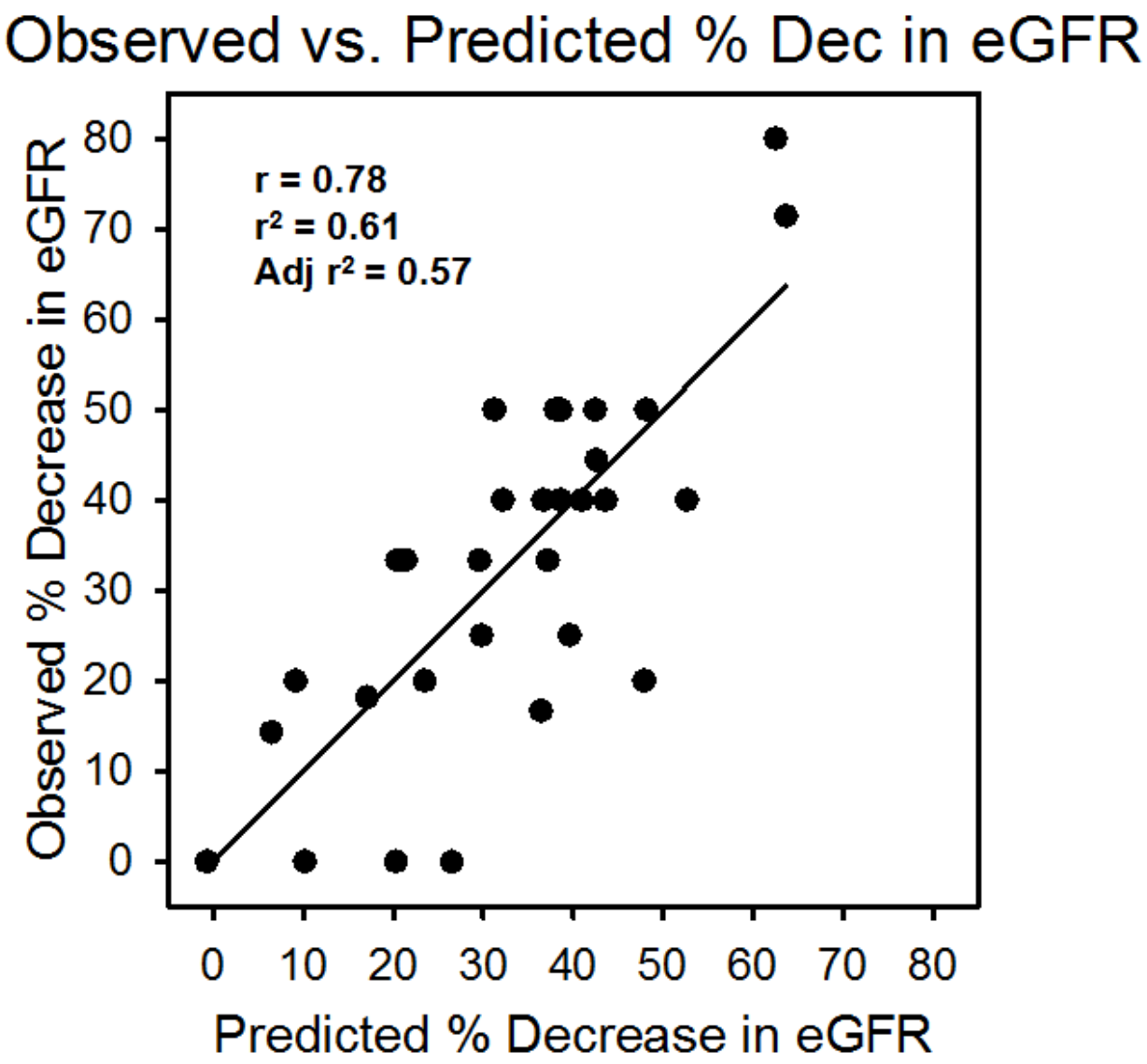


Figure 7: Multiple linear regression residual plots

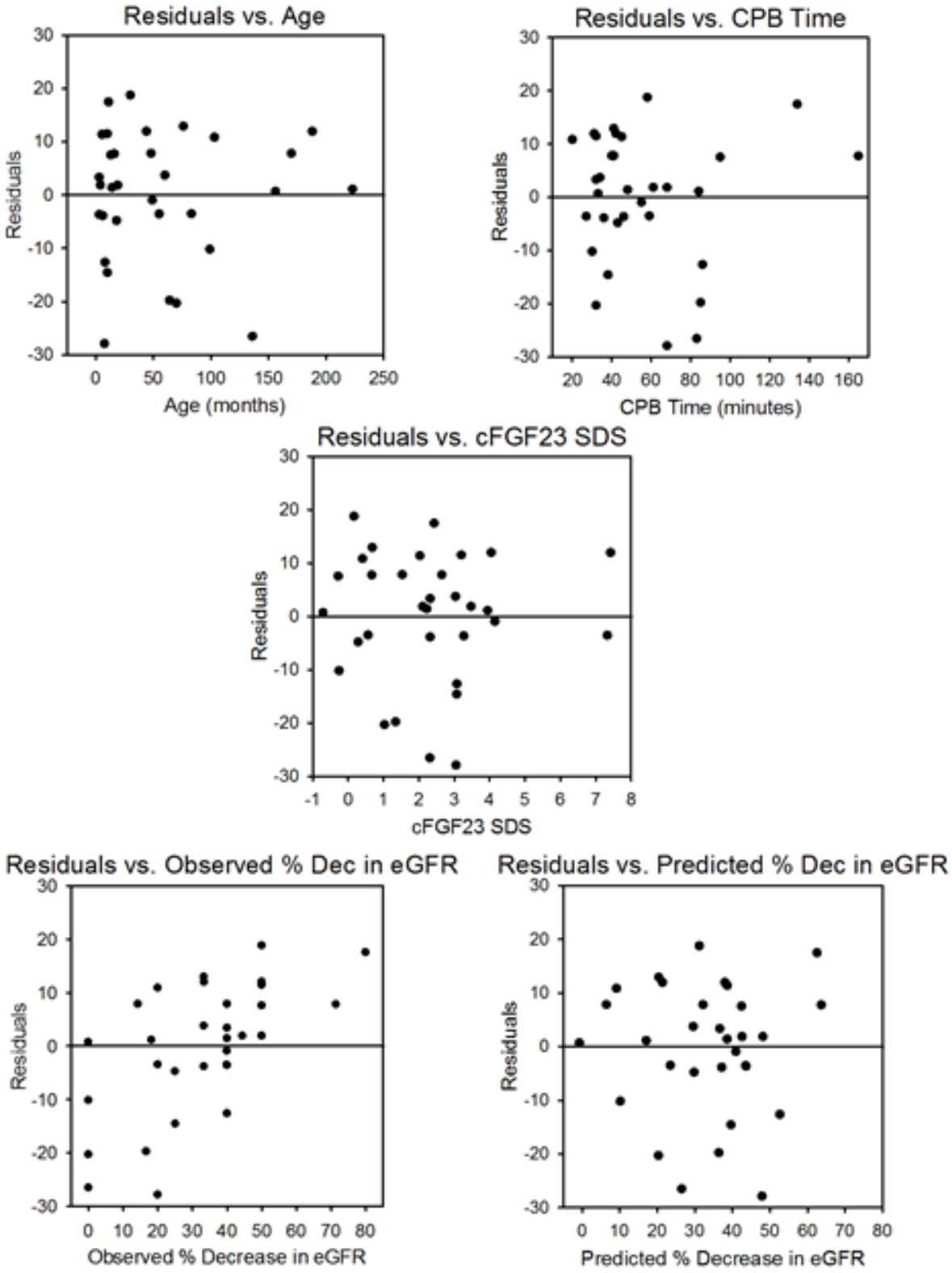


Figure 8: Multiple linear regression standardized residuals

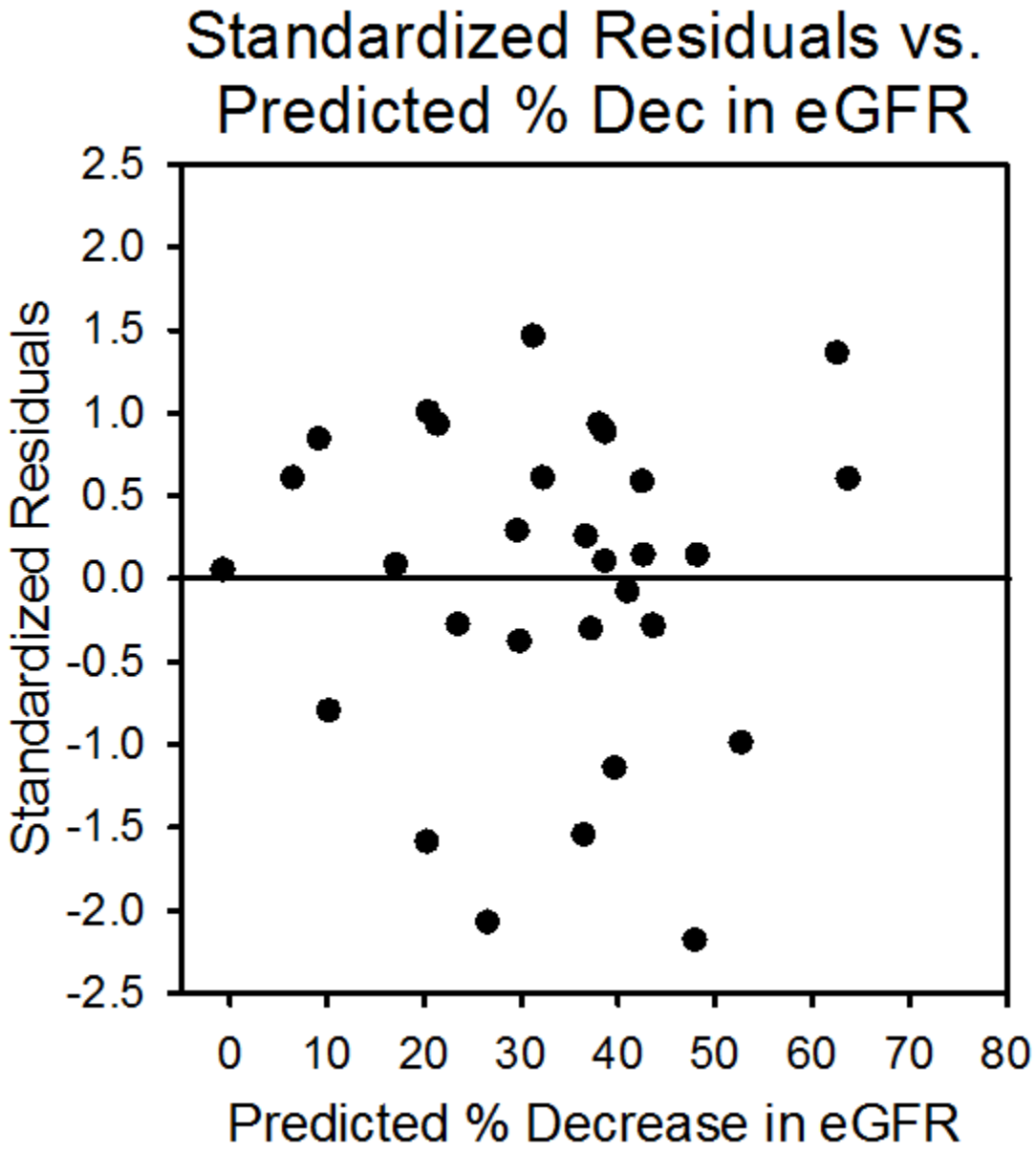


Figure 9: Multiple linear regression normal probability plot

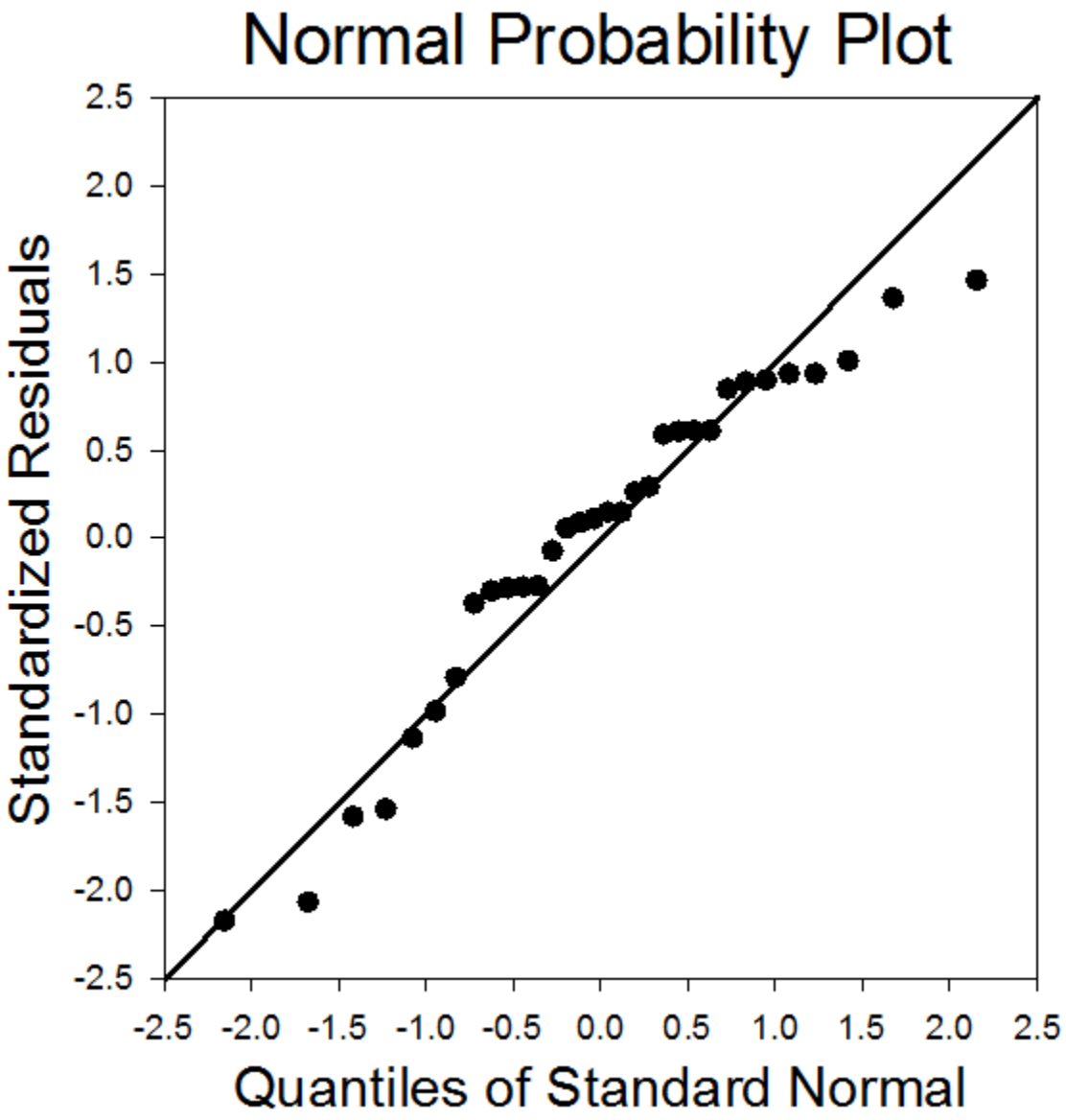


Figure 10: Multiple linear regression Studentized residuals and Studentized deleted residuals

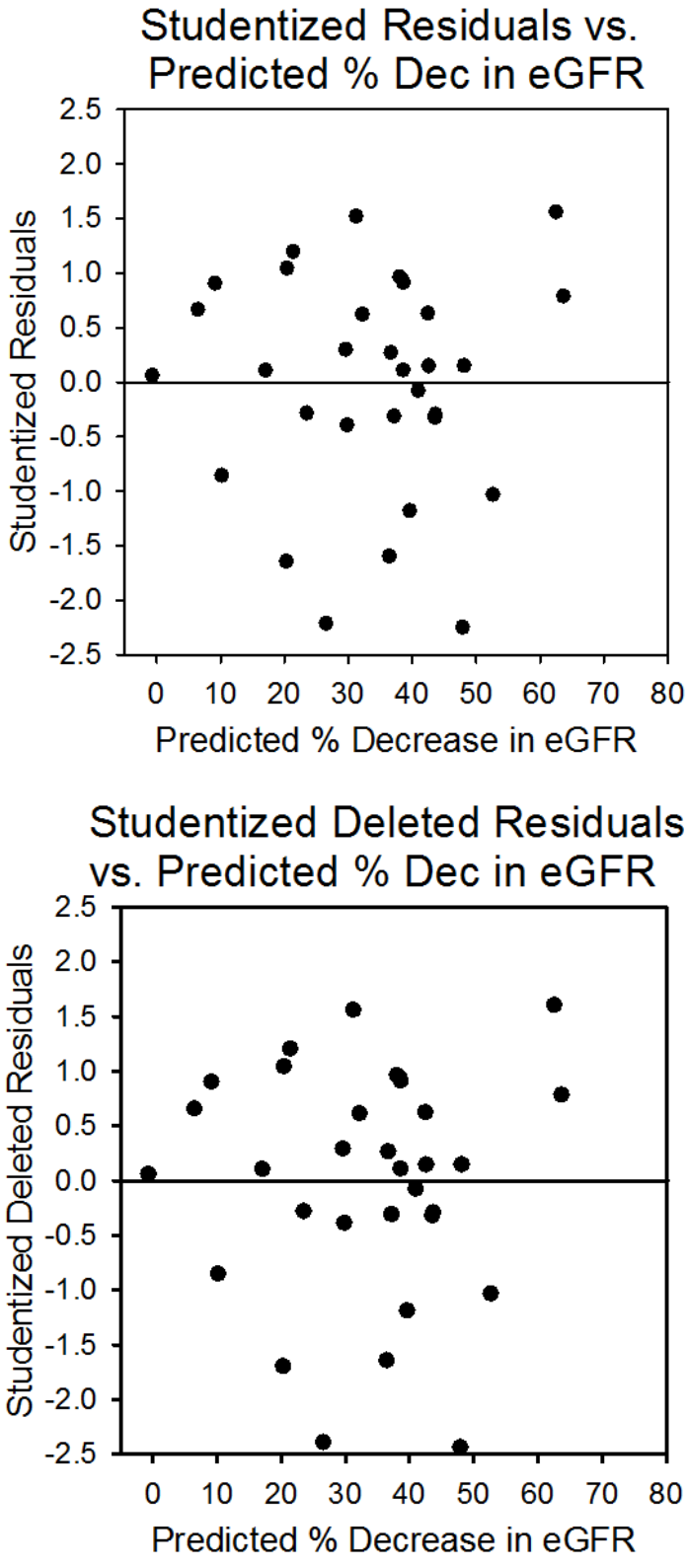


Figure 11: Multiple linear regression Studentized deleted residuals vs. leverage

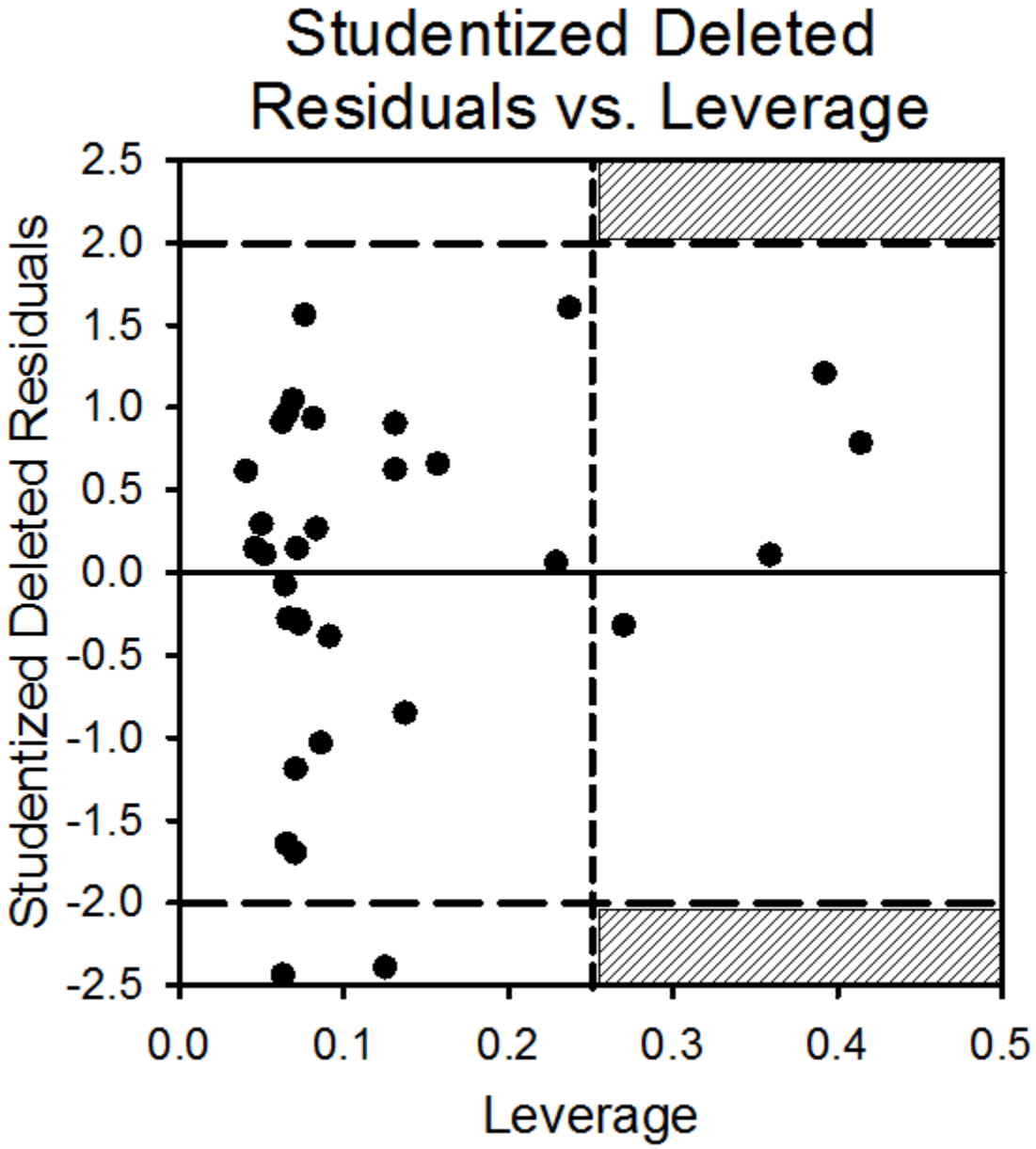
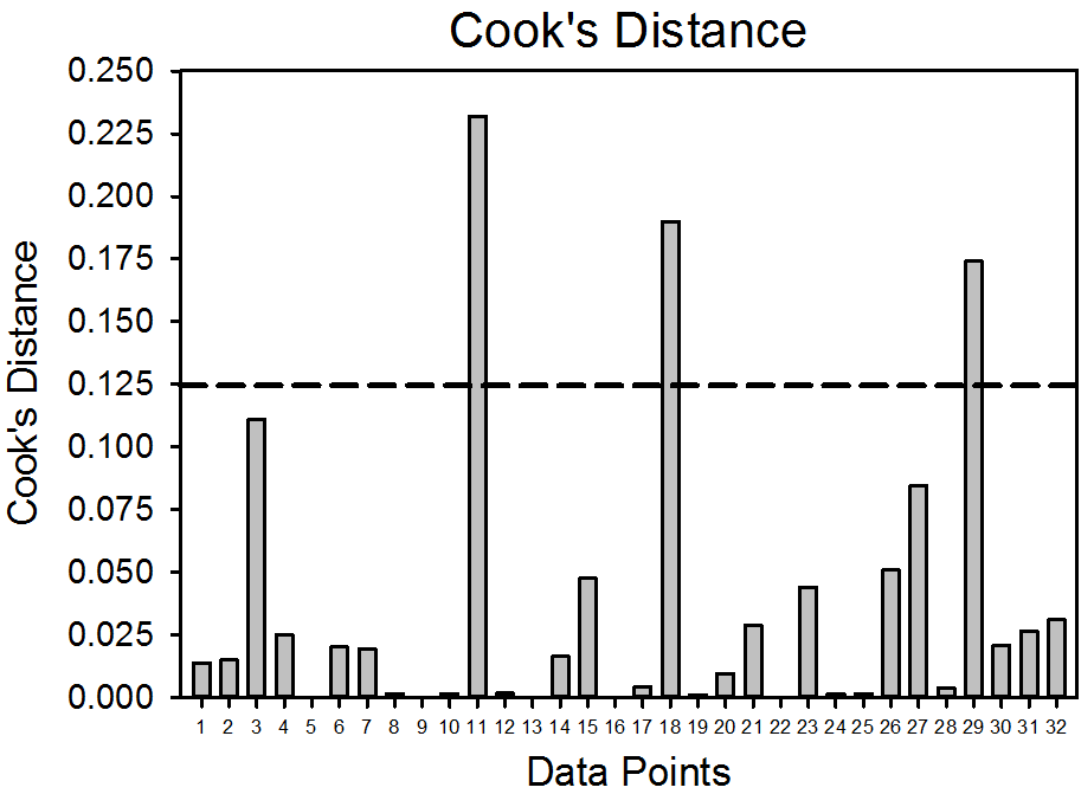


Figure 12: Multiple linear regression Cook's distances and DFFITS values



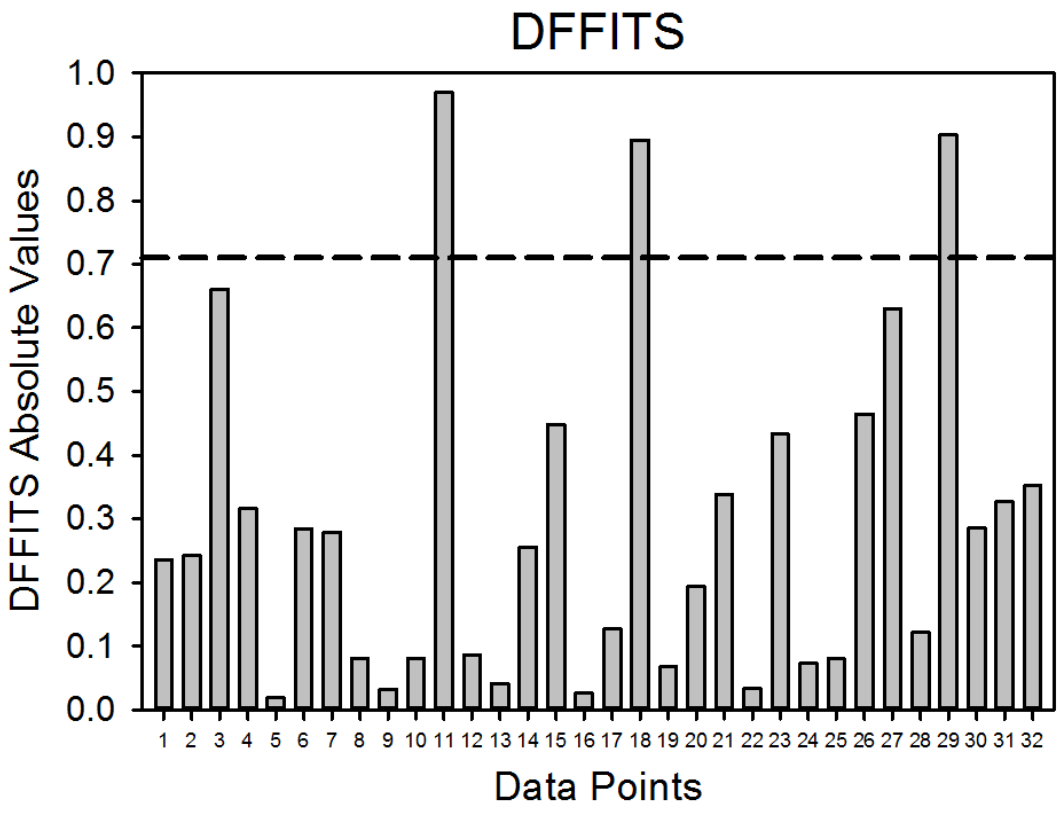
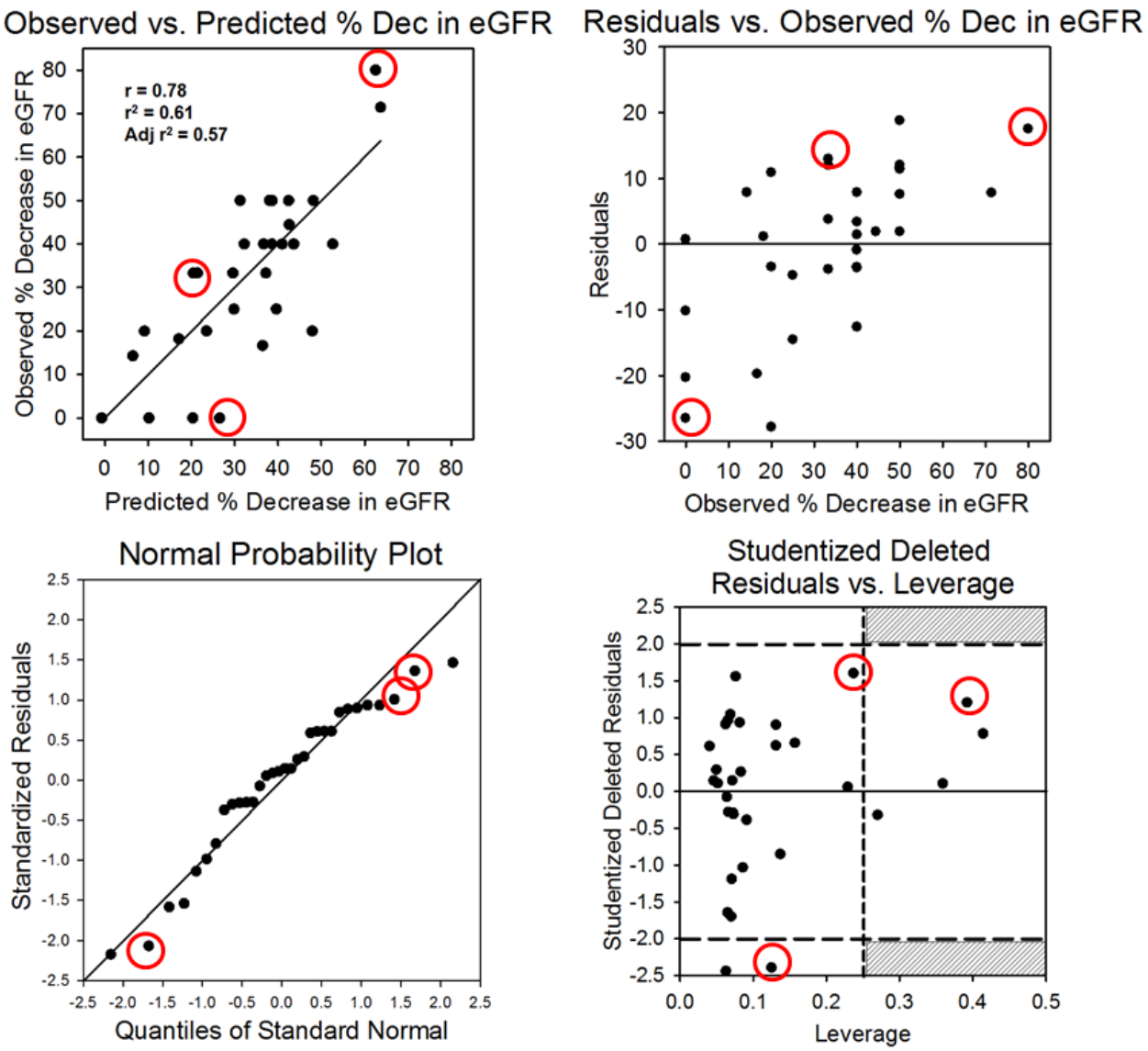


Figure 13: Multiple linear regression potentially influential observations



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