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Novel filtration markers for GFR estimation

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ABSTRACT

Creatinine-based glomerular filtration rate estimation ($eGFR_{cr}$) has been improved and refined since the 1970s through both the Modification of Diet in Renal Disease (MDRD) Study equation in 1999 and the CKD Epidemiology Collaboration (CKD-EPI) equation in 2009, with current clinical practice dependent primarily on $eGFR_{cr}$ for accurate assessment of GFR. However, researchers and clinicians have recognized limitations of relying on creatinine as the only filtration marker, which can lead to inaccurate GFR estimates in certain populations due to the influence of non-GFR determinants of serum or plasma creatinine. Therefore, recent literature has proposed incorporation of multiple serum or plasma filtration markers into GFR estimation to improve precision and accuracy and decrease the impact of non-GFR determinants for any individual biomarker. To this end, the CKD-EPI combined creatinine-cystatin C equation ($eGFR_{cr\text{-}cys}$) was developed in 2012 and demonstrated superior accuracy to equations relying on creatinine or cystatin C alone ($eGFR_{cr}$ or $eGFR_{cys}$). Now, the focus has broadened to include additional novel filtration markers to further refine and improve GFR estimation. Beta-2-microglobulin (B2M) and beta-trace-protein (BTP)

are two filtration markers with established assays that have been proposed as candidates for improving both GFR estimation and risk prediction. GFR estimating equations based on B2M and BTP have been developed and validated, with the CKD-EPI combined BTP-B2M equation ($eGFR_{BTP-B2M}$) demonstrating similar performance to $eGFR_{cr}$ and $eGFR_{cys}$. Additionally, several studies have demonstrated that both B2M and BTP are associated with outcomes in CKD patients, including cardiovascular events, ESRD and mortality. This review will primarily focus on these two biomarkers, and will highlight efforts to identify additional candidate biomarkers through metabolomics-based approaches.

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INTRODUCTION

It is currently estimated that 15% of US adults, or about 30 million people, have chronic kidney disease (CKD)[1]. CKD is defined as the presence of kidney damage, or estimated glomerular filtration rate (eGFR) < 60 mL/min/1.73 m^2, for a duration of at least 3 months[2]. Once diagnosed, CKD is staged based on cause of disease, level of GFR and albuminuria, to provide guidance for disease management and risk stratification[2]. GFR is accepted as the best overall measure of kidney function in health and disease and reflects the product of the number of nephrons and the average single nephron GFR[3]. Measured glomerular filtration rate (mGFR) via quantification of urinary or plasma clearance of an exogenous filtration marker remains the gold standard for assessing GFR in patients with CKD. However, GFR measurement is burdensome for patients as well as clinical laboratories. Therefore, clinicians instead routinely use GFR estimates to diagnose and manage patients with CKD. The 2012 Kidney Disease Improving Global Outcomes (KDIGO) guidelines recommend GFR estimation based on serum or plasma creatinine ($eGFR_{Cr}$) as the first line test, with eGFR based on cystatin C ($eGFR_{cys}$) or the combination of the two ($eGFR_{cr-cys}$) as a confirmatory test, particularly when there is concern for inaccurate $eGFR_{cr}$ results in individuals impacted by known non-GFR determinants of creatinine, such as extremes of muscle mass, a high meat-containing diet, or some dietary supplements such as creatine[4].

GFR estimating equations were developed as early as the 1970s, but it was the 4-variable Modification of Diet in Renal Disease (MDRD) Study equation developed in 2000 and re-expressed for use with standardized creatinine[5] that was the first estimating equation to become widely integrated into routine clinical laboratory reports for assessment of kidney function, due to its reliance on creatinine and readily available demographic metrics (age, gender and race)[6,7]. While this MDRD Study equation was useful for estimating GFR < 60 ml/min/1.73 m^2, it was found to systematically underestimate GFR at levels > 60 ml/min/1.73 m^2.

Therefore, the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) was formed in 2003 and set forth to improve the accuracy of GFR estimating equations by development and validation of equations based on creatinine or cystatin C in a diverse population that included participants across the range of GFR and age[3], with and without CKD, diabetes and transplants. Cystatin C was selected as a complimentary candidate filtration marker to creatinine because it is less affected by non-GFR determinants that impact creatinine[8], and several studies have demonstrated that it is a better prognostic marker for predicting development of cardiovascular disease and mortality than creatinine[9-11].

The CKD-EPI group developed and validated a new CKD-EPI creatinine-based eGFR equation, which was found to have lesser bias compared

to measured GFR at GFR > 60 than the MDRD Study equation, and therefore its use was recommended as an improvement over the MDRD Study equation[12]. This work was followed by publication of two papers which demonstrated that estimating equations which relied on both creatinine and cystatin C were superior in precision to equations that relied on only one biomarker alone[13,14]. These studies laid the groundwork for the main hypothesis driving current efforts to improve GFR estimation – that incorporation of additional biomarkers into estimation of GFR diminishes the impact of non-GFR determinants for any given biomarker and improves overall equation performance. Based on this hypothesis, research in the area of GFR estimation has moved from fine-tuning current creatinine and cystatin C-based equations to identifying new endogenous filtration markers that can be incorporated into GFR estimation to improve precision.

Beta-2-microglobulin (B2M) and beta-trace protein (BTP) have been identified as two endogenous low molecular weight protein filtration markers with established assays that have the potential to improve the accuracy of GFR estimations. Additionally, due to technologic advances in the field of metabolomics, work is currently in progress to identify and validate the utility of additional, novel filtration markers, with subsequent development of validated assays.

BETA-2-MICROGLOBULIN (B2M)

B2M is a 11.8 kD protein which associates with both classical and non-classical MHC Class I molecules on the surface of all cells and is critical for antigen presentation[15]. It is freely filtered by the glomerulus, with more than 99.9% reabsorbed and metabolized in the proximal tubule[15]. Serum/plasma B2M concentrations are impacted by the amount generated and shed by nucleated cells, body distribution kinetics, and the amount eliminated through glomerular filtration and tubular metabolism. Due to its ubiquitous presence on the surface of all cells, B2M elevation is seen with diseases associated with high cell turnover, such as many malignancies. Therefore, B2M is most commonly measured along with serum albumin to risk stratify multiple myeloma patients using the International Staging System (ISS)[16], with higher levels of B2M associated with higher tumor burden and more aggressive subtypes, due to increased shedding of B2M[15].

B2M was first suggested as a biomarker for glomerular filtration in the 1980s[17,18], however, as an acute phase reactant that increases in a variety of inflammatory and infectious disorders, its potential as a candidate for a single-marker equation was limited[19,20]. Despite this shortcoming a handful of research groups derived GFR estimating equations based on B2M alone, but data supporting the performance and validity of these equations is lacking[21-24].

Elevation of B2M in patients with CKD, especially end stage renal disease (ESRD), has been traditionally attributed to impaired removal secondary to decreased glomerular filtration. However recent literature has put forth the hypothesis that an additional source of B2M elevation in patients with CKD may be the interference of uremic solutes with the non-covalent binding of B2M to MHC molecules, leading to an increase in shedding of B2M into the circulation[15].

Due to its established use as a prognostic marker for multiple myeloma, B2M is routinely measured in many clinical laboratories by a variety of methods – nephelometry, turbidimetry, or immunoassay[25]. However, studies have demonstrated that B2M assays are not harmonized or standardized leading to discordance between methods[25,26]. While the WHO 1st International Standard for B2M was developed in 1985[27],

and a B2M certified reference value in the serum protein standard ERM-DA470k/IFCC was assigned in 2015 by the Institute for Reference Materials and Measurements (IRMM)[28], manufacturers have not universally adopted use of ERM-DA470k/IFCC for calibration of their measurement procedures[25].

BETA-TRACE PROTEIN (BTP)

BTP, also known as lipocalin prostaglandin D_2 synthase (L-PGDS), is a 23-29 kDa protein. The variation in size depends on the degree of post-translational glycosylation[29], with the larger isoforms of BTP in serum and urine, and smaller isoforms with truncated side chains in cerebrospinal fluid (CSF)[29]. BTP was first noted to be elevated in patients with CKD in 1987, as an incidental finding in a study focused on BTP as a marker for CSF leak[30]. Its specific potential as a filtration marker was not suggested until 1997, in a study that observed very high levels of BTP in patients on hemodialysis[31].

The first GFR estimating equations based on BTP were derived in 2007 by White and colleagues, in a cohort of 163 adult kidney transplant patients with measured GFR. These equations, known as the White equations, performed comparably to the MDRD Study equation, with evidence of improved performance at higher GFRs[32]. The following year, researchers led by Dr. Uwe Pöge developed 3 additional BTP-based GFR estimating equations from a cohort of 85 kidney transplant patients validated in a separate cohort of 102 kidney transplant patients[33]. The three Pöge equations were compared to the re-expressed MDRD Study equation and White equation 1 (based on BTP and urea). The Pöge BTP-formula 3 had better accuracy and precision than White equation 1, and demonstrated a slightly smaller bias and higher 10% accuracy when compared to the re-expressed MDRD Study equation[33]. The generalizability of these equations to clinical populations other than kidney transplant recipients has not been

Table 1 GFR estimating equations based on BTP developed by White[32] and Pöge[33]

Description	Development population	Equation
White Equation 1 (BTP & urea)	N = 163, kidney transplant patients	$eGFR = 112.1 \times BTP^{-0.662} \times Urea^{-0.280} \times (0.880 \text{ if female})$
White Equation 2 (BTP & Cr)	N = 163, kidney transplant patients	$eGFR = 167.8 \times BTP^{-0.758} \times Cr^{-0.204} \times (0.871 \text{ if female})$
Pöge BTP-formula 1 (BTP alone)	N = 85, kidney transplant patients	$eGFR = 47.17 \times BTP^{-0.7933}$
Pöge BTP-formula 2 (BTP & Cr)	N = 85, kidney transplant patients	$eGFR = 974.31 \times BTP^{-0.2594} \times Cr^{-0.647}$
Pöge BTP-formula 3 (BTP & urea)	N = 85, kidney transplant patients	$eGFR = 89.85 \times BTP^{-0.5541} \times Urea^{-0.3018}$

White and Pöge formulas utilize units of mg/L for BTP, mmol/L for creatinine, and mmol/L for urea.

established, and these minor differences were not deemed sufficient enough to recommend replacement of the MDRD Study equation for routine clinical practice[33]. (Table 1)

While GFR estimating equations based on BTP appear promising, a major hurdle involves the lack of standardization amongst currently available BTP assays[29,34]. Unlike creatinine, cystatin C and B2M, there are currently no certified reference materials available for BTP. Additionally, given the known variation in post-translational modification which creates a variety of glycoprotein epitopes, immunoassays utilizing different antibodies would be expected to give disparate BTP results.

USING BTP AND B2M TO IMPROVE GFR ESTIMATION

Given the various shortcomings of relying on BTP or B2M alone for GFR estimation, the CKD-EPI investigators evaluated the utility of combining the markers[35]. Data was pooled from 3 separate research studies involving a total of 3,551 subjects with CKD due to a variety of causes, each with GFR measured based on urinary clearance of iothalamate[35]. Equations were developed using either BTP or B2M concentrations alone and in combination (Table 2).

The performance of the three equations was compared to the CKD-EPI creatinine- and cystatin C-based equations based on precision (Table 3). Their analysis demonstrated that the combined BTP-B2M equation had similar performance to both the creatinine and cystatin C equations but did not represent an improvement over either equation[35]. Additionally, the combined BTP-B2M equation was not as accurate as the combined creatinine-cystatin C equation[35]. Lastly, averaging the BTP-B2M equation with the creatinine-cystatin C equation did not lead to improvement in equation performance[35]. Limitations of this work included the absence of participants without CKD and an external validation population. (Table 3)

While the non-GFR determinants of creatinine were already well-established, it was important to more fully characterize the non-GFR determinants of cystatin C, BTP and B2M. Preliminary studies demonstrated evidence for non-GFR determinants of cystatin C, including inflammation, immunosuppressive therapies, thyroid disease and obesity[36-39], but there were few studies that had evaluated the non-GFR determinants of BTP and B2M. Therefore, in 2016 the CKD-EPI investigators published a cross-sectional analysis of these same CKD cohorts which characterized the non-GFR determinants for these three biomarkers[40]. Their analysis showed that

Table 2 CKD-EPI BTP and B2M equations[35]

Description	Development population	Equation
BTP	N = 2,380, chronic kidney disease patients	$GFR = 55 \times BTP^{-0.695} \times 0.998^{age} \times 0.899$ if female
B2M	N = 2,380, chronic kidney disease patients	$GFR = 133 \times B2M^{-0.852}$
BTP-B2M	N = 2,380, chronic kidney disease patients	$GFR = 96 \times BTP^{-0.278} \times B2M^{-0.588}$

Table 3 Performance of CKD-EPI GFR Estimating Equations (Adapted from Inker et al.[35])

Description	Inter-quartile range (95% CI)	1-P_{30} (%)(95% CI)	1-P_{20} (%) (95% CI)
BTP	15.0 (14.1, 15.9)	23.6 (21.3, 26.1)*	43.6 (40.8, 46.5)
B2M	12.9 (12.2, 13.8)	18.4 (16.2, 20.8)*	37.2 (34.6, 40.1)
BTP-B2M	12.1 (11.4, 13.0)	15.5 (13,3, 17.7)*	35.4 (32.5, 38.1)
Creatinine	11.6 (10.9, 12.4)	16.4 (14.2, 18.6)*	34.5 (31.7, 37.3)
Cystatin C	11.4 (10.6, 12.4)	16.9 (14.9, 18.6)*	34.8 (32.1, 37.6)
Creatinine-Cystatin C	9.3 (8.7, 10.1)	11.3 (9.5, 13.2)	25.5 (23.1, 28.0)
Average of Creatinine-Cystatin C + BTP-B2M	10.2 (9.5, 11.0)	9.6 (8.0, 11.4)	25.0 (22.6, 27.6)

P_{30} and P_{20} are the percentage of GFR estimates > 30% and > 20% from measured GFR
**P < 0.001 when compared to the creatinine-cystatin C equation*

Table 4 Summary of major non-GFR determinants for filtration markers[40,41]

GFR biomarker	Non-GFR determinant profile
Creatinine	Male sex, black race, elevated urine creatinine, age
Cystatin C	Male sex[1], smoking, body mass index (BMI) and C-reactive protein (CRP)
BTP	Male sex[1], urine protein excretion, non-black race, body mass index (BMI)
B2M	Urine protein excretion, smoking and C-reactive protein (CRP)

[1]The association between male sex and creatinine was stronger than the associations between male sex and BTP or cystatin C

creatinine was more strongly associated with male sex, black race and elevated urine creatinine than BTP, B2M or cystatin C. In addition, each filtration marker exhibited unique profiles of non-GFR determinants (Table 4).

In 2017, non-GFR determinants of these filtration markers were further characterized in 2 community-based, predominantly elderly cohorts (Table 4)[41]. Again, creatinine was found to more strongly associate with age and sex than

cystatin C, BTP or B2M. Additionally, both cystatin C and B2M had significant associations with CRP, confirming prior studies demonstrating a relationship between inflammation and inflammatory diseases and these biomarkers[15,42]. Not all associations were duplicated between the two studies, and therefore more research is needed. Both studies did provide evidence that each filtration marker has unique non-GFR determinant profiles, providing a foundation of support for the hypothesis that combining multiple markers with differing non-GFR determinants for GFR estimation has the potential to minimize bias and imprecision, thereby improving accuracy. Additionally, $eGFR_{cys}$, $eGFR_{BTP}$ and $eGFR_{B2M}$ were less influenced by race than $eGFR_{cr}$, thus introducing the possibility of developing a multiple marker estimating equation without creatinine which would eliminate the need for race specification.

PROGNOSTIC VALUE OF BTP AND B2M

Like cystatin C, BTP and B2M are promising biomarkers in CKD not only due to their potential role in improving GFR estimation, but also due to their role as prognostic indicators. Patients with CKD have a significantly increased risk for cardiovascular disease, hospitalization and mortality compared to the general population, and therefore there is interest in predicting these outcomes[43-45].

In 2005, cystatin C was found to be a stronger predictor of mortality and cardiovascular outcomes than creatinine and $eGFR_{cr}$[10]. Additionally there is a marked discordance in mortality prediction between $eGFR_{cr}$ and $eGFR_{cys}$ at higher eGFRs, with higher $eGFR_{cr}$ associated with increased mortality while higher $eGFR_{cys}$ is associated with decreased mortality[46]. This discordance is thought to be due to non-GFR determinants of creatinine such as muscle wasting that would confound its association with outcomes in individuals in poor health, but could also be due to confounding by non-GFR determinants of cystatin C [46]. These results raised the question of whether B2M and BTP have prognostic value beyond creatinine or $eGFR_{cr}$ alone[10]. The first study proposing B2M as a prognostic marker was published in 2008, and demonstrated that B2M was an independent predictor of overall mortality in a community-based elderly population[47]. BTP was first proposed as a prognostic marker in a 2010 study which found that it was a strong predictor for future CKD progression[48].

Based on the promise of these initial studies, in 2012 researchers took a more comprehensive look at BTP and B2M as prognostic markers, by examining their association with risks for mortality, cardiovascular disease and kidney failure in a large group of subjects from the Atherosclerosis Risk in Communities (ARIC) study (n = 9,988), a middle-aged general population cohort[49].

The study found that, similar to cystatin C, B2M is a stronger predictive marker than $eGFR_{cr}$ for outcomes such as cardiovascular disease, kidney failure and mortality[49]. BTP levels also predicted these outcomes more strongly than $eGFR_{cr}$, although not to the degree of cystatin C and B2M levels[49]. This study was followed up by a similar analysis performed on 6,445 subjects from the Third National Health and Nutrition Examination Survey (NHANES III), a general population cohort spanning the range of adulthood, ages 20 and older[50]. This study also demonstrated that BTP and B2M were stronger prognostic markers than $eGFR_{cr}$, for all-cause mortality, cardiovascular disease, and coronary heart disease mortality[50]. Additionally, incorporating 4 markers – creatinine, cystatin C, B2M and BTP – into a risk prediction model led to moderate improvement in 10-year risk prediction compared to $eGFR_{cr}$, when adjusted for mortality and cardiovascular risk factors[50].

While these studies supported the utility of B2M and BTP as prognostic markers in the general population, they did not examine their utility in clinically relevant sub-populations, such as diabetics or patients with chronic kidney disease, or in racial groups other than Whites or African-Americans. Therefore, their role as risk predictors in a type 2 diabetic Pima Indian cohort was examined in 2015[51].

This study found that both BTP and B2M were associated with ESRD, with BTP having the stronger association[51]. Interestingly in this study only B2M, and not BTP, was associated with mortality, after adjustment for other mortality risk factors and kidney function markers[51]. Therefore, B2M may be a more useful prognostic marker than BTP in this subpopulation of Pima Indian diabetics. To further address the potential role of BTP and B2M in clinically significant subpopulations, a cohort of CKD patients was examined to specifically look at B2M and BTP's role in predicting cardiovascular events, ESRD and mortality[52]. This study demonstrated that both B2M and BTP were independently associated with ESRD and all-cause mortality, and B2M was associated with risk for cardiovascular events in these patients with mild or moderate CKD[52]. Additionally, a 4-marker composite score generated from $eGFR_{cr}$, $eGFR_{cys}$, B2M and BTP levels was independently associated with all three outcomes – ESRD, all-cause mortality and cardiovascular events[52]. Of note, this analysis showed that BTP and B2M are associated with ESRD, and B2M and the 4-marker composite score were significantly associated with all-cause mortality and cardiovascular events even after adjustment for mGFR, indicating that non-GFR determinants contribute to risk prediction[52]. These findings support prior studies that have shown that B2M or BTP have prognostic value beyond measured GFR[51,53].

Lastly, a recent individual patient meta-analysis from the CKD Biomarkers Consortium study also examined the association between eGFR based on the four filtration markers (creatinine, cystatin C, BTP and B2M) alone and in combination with each other, through analysis of the three cohorts described above (ARIC, NHANES III, Pima) combined with three CKD study populations–Chronic Renal Insufficiency Cohort (CRIC), Modification of Diet in Renal Disease (MDRD) study and African American Study of Kidney Disease (AASK)[54].

Consistent with the data supporting association of B2M and BTP with risk outcomes, this study found that $eGFR_{B2M}$ and $eGFR_{BTP}$ modestly improved prediction of ESRD and mortality over $eGFR_{Cr}$[54]. Additionally, this meta-analysis demonstrated that higher $eGFR_{B2M}$ and $eGFR_{BTP}$ are associated with lower mortality, similar to $eGFR_{cys}$[54], consistent with the hypothesis that increased mortality associated with higher $eGFR_{cr}$ reflects confounding by non-GFR determinants of creatinine such as muscle wasting in patients in poor health. Additionally, eGFR based on the average of the estimated GFRs from all 4 biomarkers provided the best overall performance for risk prediction, albeit only a modest improvement over $eGFR_{Cr}$[54].

This study and others together demonstrate that combining multiple filtration markers provides the best overall performance for predicting risk outcomes.

LOOKING TO THE FUTURE —KIDNEY METABOLOMICS

Advances over the last decade in mass spectrometry and associated chromatography methods have led to an explosion of metabolomics studies aimed at discovering novel biomarkers for various diseases[55].

In 2010, the first targeted metabolomics studies in CKD patients identified novel uremic toxins, but the studies were too limited in size and power to draw firm conclusions about the identified metabolites[56-58].

In 2012, the first large-scale targeted metabolomics study in subjects spanning the range of GFR was performed using 3,011 samples from the KORA F4 study for metabolite discovery, and 984 samples from the TwinsUK study for metabolite validation[59]. A total of 22 metabolites and 516 metabolite ratios were identified as having a significant association with $eGFR_{cr}$, with acylcarnitines having the strongest association[59].

This cross-sectional analysis was soon followed by a targeted longitudinal metabolomics study in 2013, aimed at determining whether the same or different metabolites and metabolite ratios were associated with development of $eGFR_{cr}$ decline over time independent of baseline eGFR[60]. The study examined associations between 140 metabolites and 19,460 ratios with the incidence of decreased $eGFR_{cr}$ and $eGFR_{cr}$ decline over a 7 year period in 1,104 subjects from the KORA study[60]. This longitudinal analysis demonstrated that the acylcarnitines overall did not significantly associate with $eGFR_{cr}$ decline over time. Rather, the study identified one metabolite and two ratios that had a significant association with change in $eGFR_{cr}$ over time – spermidine, the kynurenine-to-tryptophan ratio, and the phosphatidylcholine diacyl C42:5-to-phosphatidylcholine acylalkyl C36:0 ratio – all of which were supported by smaller, prior studies[61-64].

In 2016, the first large-scale non-targeted metabolomics study was published, with metabolite discovery performed on samples from 1735 Kora study subjects, and validated in 1164 samples from the TwinsUK study[65]. A non-targeted approach has the advantage of identifying previously unrecognized CKD-associated metabolites. Of the 493 small molecules quantified in the study, 54 metabolites had a validated significant association with $eGFR_{cr}$, with 6 metabolites demonstrating a significant pairwise correlation: C-mannosyltryptophan, pseudouridine, N-acetylalanine, erythronate, myo-inositol and N-acetylcarnosine[65]. Additionally, three metabolites (C-mannosyltryptophan, pseudouridine, and O-sulfo-L-tyrosine) were significantly associated with development of low $eGFR_{cr}$[65]. Studies comparing metabolites to measured GFR have been reported and could yield more accurate estimates of GFR whose generalizability and robustness will need to be tested.

CONCLUSION

While there have been marked improvements in the accuracy of GFR estimation using serum- or plasma-based biomarkers over the last 20 years with refinement of equations based on creatinine and cystatin C, inaccuracy of estimated GFR remains a challenge due to the impact of non-GFR determinants of these biomarkers. B2M and BTP hold promise as candidate endogenous filtration markers that have the potential to improve the accuracy of both GFR estimation and risk prediction.

Additionally, cystatin C, B2M and BTP are less affected by race than creatinine, and therefore provide the potential opportunity to estimate GFR without the need for race specification. Kidney metabolomics research is in the early phases of metabolite discovery and validation, with work on the horizon to assess the clinical feasibility of using additional, new biomarkers for improved GFR estimation and risk prediction. Thus, the focus is shifting to the concept of estimating GFR with a panel of several serum or plasma biomarkers, to minimize the impact of each individual biomarker's non-GFR determinants.

Additionally, multiple studies on BTP and B2M as prognostic markers support the idea that risk prediction also improves when multiple markers are combined. Therefore, novel biomarkers identified via metabolomics profiling in chronic kidney disease patients will likely be combined with biomarkers such as creatinine, cystatin C, B2M and BTP, for future incorporation into multi-biomarker estimating equations for GFR and multi-biomarker risk prediction models.

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