Effects of Traumatic Amputation on β -Trace Protein and β_2 -Microglobulin Concentrations in Male Soldiers

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Key Words

Amputation · Beta 2 microglobulin · Beta trace protein · Glomerular filtration rate estimation

Abstract

Background: Serum creatinine (SCr) levels are decreased following traumatic amputation, leading to the overestimation of glomerular filtration rate (GFR). β -Trace protein (BTP) and β_2 -microglobulin (B2M) strongly correlate with measured GFR and have not been studied following amputation. We hypothesized that BTP and B2M would be unaffected by traumatic amputation. **Methods:** We used the Department of Defense Serum Repository to compare pre- and post-traumatic amputation serum BTP and B2M levels in 33 male soldiers, via the N Latex BTP and B2M nephelometric assays (Siemens Diagnostics, Tarrytown, N.Y., USA). Osterkamp estimation using DEXA scan measurements was used to establish percent estimated body weight loss (%EBWL). Results were analyzed for small (3–5.9% EBWL), medium (6–13.5%), and large (>13.5%) amputation subgroups; and for a control group matched 1:1 to the 12 large amputation subjects. Paired Student's t test was used for comparisons. **Results:**

Mean serum BTP levels were unchanged in controls, all amputees, and the small and medium amputation subgroups. BTP appeared to decrease following large %EBWL amputation ($p = 0.05$). Mean serum B2M levels were unchanged in controls, all amputees, and the small and medium amputation subgroups. B2M appeared to increase following large %EBWL amputation ($p = 0.05$). **Conclusions:** BTP and B2M levels are less affected than SCr by amputation, and should be considered for future study of GFR estimation. BTP and B2M changes following large %EBWL amputation require validation and may offer insight into non-GFR BTP and B2M determinants as well as increased cardiovascular disease and mortality following amputation.

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Introduction

New estimating equations based on serum creatinine (SCr) and SCr combined with serum cystatin C (SCysC) enable improved glomerular filtration rate (GFR) esti-

Fig. 1. Flowsheet showing results of application of exclusion criteria to 91 subjects identified by screening.

mation [1]. But, there are exceptions, such as traumatic amputation, where these equations could be less accurate [2, 3]. Traumatic amputation is associated with combat, natural disasters, accidents, and acts of violence. Trauma accounts for 16% of US amputation incidence, but comprises 45% of prevalent limb loss cases [4]. Over 1,500 US service members have sustained battle-injury major limb amputations in the Iraq and Afghanistan wars [5], and an estimated 900,000 Americans will be living with traumatic amputation in 2020 [4]. Rates of cardiovascular disease, cardiovascular mortality, and overall mortality are increased following traumatic amputation [6–11]. Undiagnosed CKD due to overestimation of GFR by SCr-based equations could be one explanation, since high rates of proteinuria have been reported following apparent recovery from battle injury-associated post-traumatic acute kidney injury (AKI) [12], traumatic amputation can cause significant loss of creatinine-producing skeletal muscle, and CKD patients suffer from increased rates of cardiovascular disease and mortality [13, 14].

We previously demonstrated that cystatin C was superior to SCr for the estimation of GFR following amputation [2]. However, cystatin C is produced by all nucleated cells and there was a small difference in cystatin C following a large amputation ($p = 0.21$) that may have been statistically significant with a larger study group [2]. The filtration markers β -trace protein (BTP) and β_2 -microglobulin (B2M) strongly correlate with measured GFR (mGFR) and subsequent cardiovascular disease and mortality [15–19]. We hypothesized that serum BTP would be a superior filtration marker for GFR estimation after amputation because it is primarily produced in the brain and heart [20–23] as opposed to muscle (SCr) or all nucleated cells (cystatin C). In the general population, GFR estimation is improved by incorporation of multiple

filtration markers [1]. Therefore, we sought to determine if BTP and B2M are candidates to improve GFR estimation in amputees.

Subjects and Methods

Patients

We conducted a Department of Defense Serum Repository (DoDSR) study comparing pre and post-traumatic amputation BTP and B2M levels, using serum specimens from which pre- and post-amputation SCr and SCysC levels had been previously quantified [2]. The study was approved by the Walter Reed National Military Medical Center institutional review board. The study was deemed minimal risk, and a waiver of informed consent was obtained. Potential subjects were identified by searching the Armed Forces Amputee Care Program Database. Subjects were previously healthy 18–50-year-old active duty male soldiers who sustained amputation of $\geq 3\%$ estimated body weight loss (%EBWL) within 12 weeks of initial trauma. Extent %EBWL was determined by Osterkamp [24] and Mozumdar and Roy [25] estimations from dual-energy X-ray absorptiometry. Exclusion criteria included staged amputations; body mass index $> 35 \text{ kg/m}^2$; chronic kidney disease defined as $\text{eGFR} < 60 \text{ ml/min/1.73 m}^2$, urine albumin-to-creatinine ratio $> 30 \text{ mg/g}$ creatinine, or urine protein-to-creatinine ratio of $> 0.3 \text{ mg/mg}$ creatinine; burns, chronic infection, malignancy, malnourishment, cardiovascular disease, cerebrovascular disease, lack of serum in the DoDSR; and AKI with any evidence of proteinuria defined as $\geq 1+$ on urinalysis or urine protein-to-creatinine ratio of $> 0.3 \text{ mg/mg}$ creatinine, obstruction, acute tubular necrosis, kidney trauma, or failure of SCr to return to $< 1.5 \text{ mg/dl}$ within 72 h of volume resuscitation. Records of 91 individuals were screened, and 33 subjects met inclusion criteria (fig. 1). A comprehensive data-collection sheet was populated for each study subject, using data obtained from electronic medical record review. The study cases were divided into subgroups of 9 small (3–5.9%), 12 medium (6–13.5% EBWL), and 12 large ($> 13.5\%$ EBWL) amputation subjects. Thresholds were based on common amputation levels ranging from unilateral trans-knee amputation (small) to unilateral trans-femoral amputation or bilateral trans-knee amputation (medium) to bilateral trans-femoral amputation (large). The DoDSR identified a pre (< 24 months prior to amputation) and post (preferentially 2–24 months following amputation, up

Table 1. Characteristics of amputee study population and controls

Variables	All amputees (n = 33)	Small (n = 9)	Medium (n = 12)	Large (n = 12)	Controls (n = 12)
Age, years	24 (20–38)	27 (21–38)	24 (20–33)	23 (20–32)	24 (21–32)
Race					
Black	3	1	2	–	1
White	25	6	7	11	10
Other	5	2	3	1	1
Date of amputees	9/27/2006–9/15/2011	10/18/2006–4/09/2011	9/27/2006–2/17/2011	6/16/2007–9/15/2011	NA
Mechanism	25 IED, 1 RPG, 2 MVA, 2 NS	7 IED, 1 RPG, 1 MVA	10 IEDs, 1 MVA, 1 NS	11 IED, 1 NS	NA
%EBWL	12 (4–24)	5 (4–6)	10 (8–13)	19 (14–24)	NA
BMI					
Pre	24.9 (20.7–29.4)	24.7 (20.7–28.7)	25.7 (21.8–29.4)	24.3 (22.3–27.5)	NA
Post	22.7 (16.0–29.4)	25.1 (19.7–28.5)	23.7 (16.0–29.4)	19.8 (16.2–25.9)	NA
Post (A)BMI	25.7 (18.2–32.7)	26.0 (20.5–29.6)	27.2 (22.5–32.7)	23.7 (18.2–28.5)	NA
TBI (severity)	14 (13 mild, 1 mod.)	0	5 (4 mild, 1 mod.)	9 (9 mild)	NA
NSAID use, %	95	100	92	92	NA
Received contrast, %	45	22	50	58	NA
Timing of serum collection, days					
Pre	247 (70–717)	284 (125–502)	190 (82–317)	278 (70–717)	273 (61–700)
Post	326 (120–1,106)	372 (121–817)	302 (124–1,106)	316 (120–909)	354 (50–1,249)
Peri-amputation albumin	2.2 (1.4–3.8)	2.7 (1.4–3.8)	2.0 (1.6–2.5)	2.0 (1.5–2.5)	NA
>30 days albumin	3.9 (2.8–4.8)	4.1 (3.0–4.8)	3.9 (2.8–4.7)	3.7 (2.8–4.4)	NA
>30 days WBC	5.2 (2.9–7.6)	5.0 (2.9–6.3)	4.7 (3.6–6.5)	5.8 (4.8–7.6)	NA

Population/controls included no women. Amputees categorized by %EBWL: small, 4–6%; medium; 6–13.5%; large, >13.5%; values in parentheses are ranges. NSAID use is percentage of patients with prescription for NSAID while hospitalized following injury. Received contrast is percentage of patients given iodinated contrast while hospitalized following injury. Timing of collections for controls is referent to date of amputation for each control patient's 1:1 matched large amputation subgroup case. No intergroup differences in albumin or WBC count were statistically significant.

(A)BMI = (Adjusted) body mass index; IED = improvised explosive device; MVA = motor vehicle accident; NS = not specified; NSAID = nonsteroidal anti-inflammatory drug; RPG = rocket-propelled grenade; mod. = moderate.

to 48 months following amputation accepted) amputation serum sample censored for time periods of hospital admission and use of trimethoprim, aminoglycoside, colistin, corticosteroid, or cimetidine. Traumatic brain injury (TBI) status and severity were determined by review of TBI screening obtained during post-amputation hospitalization. The DoDSR identified 12 healthy controls matched 1:1 to the large amputation subgroup by age, sex, age of serum samples, and date of deployment. Healthy controls were screened by ICD-9 code, and excluded if they had any chronic disease to include hypertension, chronic kidney disease, infection, or malignancy.

Laboratory Assays

Serum BTP and B2M were measured on the Siemens BN2 nephelometer using N Latex BTP and B2M assays (Siemens Diagnostics, Tarrytown, N.Y., USA) at the National Institutes of Health Clinical Center. The interassay coefficients of variation for the BTP and B2M assays were 1.6 and 3.3%, respectively. Investigators performing the assays were blinded as to whether samples were from subjects or controls.

Statistical Analysis

Demographic and clinical characteristics of cases and controls are presented as mean $\pm$ SD, median (range), counts, and proportions converted to percentages. Paired Student's *t* test performed in GraphPad QuickCalcs (GraphPad software) was used to com-

pare the mean initial and follow-up BTP and B2M concentrations for all amputees, all controls, and each subgroup. A *p* value of <0.05 was used to determine significance.

Results

Subject demographics, mechanisms of injury, and exposure to nephrotoxins are shown in table 1. Subjects were male, aged 24 ± 4 years, and 71% were white.

β -Trace Protein

Serum BTP was unchanged overall following amputation (0.57 ± 0.18 mg/l pre vs. 0.55 ± 0.16 mg/l post amputation, *p* = 0.45), in controls (0.52 ± 0.01 vs. 0.53 ± 0.08 mg/l, *p* = 0.77), and in small (0.51 ± 0.19 vs. 0.49 ± 0.19 mg/l, *p* = 0.77) and medium (0.55 ± 0.14 vs. 0.57 ± 0.16 mg/l, *p* = 0.64) %EBWL amputation subgroups (table 2; fig. 2a). Serum BTP levels appeared to decrease in the large amputation subgroup (0.65 ± 0.18 mg/l pre vs. 0.57 ± 0.15 mg/l post amputation, *p* = 0.05; table 2; fig. 2b).

Table 2. Cr, CysC, BTP, and B2M values before and after amputation

	Before amputation	After amputation	p value
SCr, mg/dl*			
All amputees	1.05±0.18 (0.76–1.53)	0.84±0.14 (0.65–1.19)	<0.001
Small amputation	1.08±0.23 (0.84–1.53)	0.94±0.14 (0.65–1.19)	0.15
Medium amputation	1.10±0.17 (0.76–1.26)	0.84±0.12 (0.65–1.06)	<0.001
Large amputation	0.97±0.12 (0.79–1.19)	0.76±0.11 (0.65–1.08)	<0.001
Controls	1.02±0.16 (0.81–1.31)	1.06±0.16 (0.84–1.37)	0.6
SCysC, mg/l*			
All amputees	0.80±0.10 (0.60–1.02)	0.79±0.08 (0.62–0.93)	0.55
Small amputation	0.75±0.10 (0.60–0.98)	0.77±0.07 (0.67–0.91)	0.60
Medium amputation	0.81±0.10 (0.66–1.02)	0.80±0.07 (0.71–0.91)	0.92
Large amputation	0.84±0.08 (0.71–0.97)	0.79±0.11 (0.62–0.93)	0.21
Controls	0.77±0.10 (0.65–0.96)	0.79±0.09 (0.63–0.93)	0.56
BTP, mg/l			
All amputees	0.57±0.18 (0.19–1.02)	0.55±0.16 (0.21–0.88)	0.45
Small amputation	0.51±0.19 (0.27–0.83)	0.49±0.19 (0.21–0.79)	0.77
Medium amputation	0.55±0.14 (0.19–0.72)	0.57±0.16 (0.27–0.79)	0.64
Large amputation	0.65±0.18 (0.41–1.02)	0.57±0.15 (0.38–0.88)	0.05
Controls	0.52±0.10 (0.29–0.63)	0.53±0.08 (0.38–0.64)	0.77
B2M, mg/l			
All amputees	1.56±0.30 (1.17–2.54)	1.60±0.30 (1.02–2.42)	0.42
Small amputation	1.44±0.29 (1.17–2.08)	1.48±0.26 (1.02–1.96)	0.74
Medium amputation	1.68±0.36 (1.29–2.54)	1.61±0.28 (1.22–2.19)	0.49
Large amputation	1.52±0.21 (1.18–1.92)	1.68±0.33 (1.29–2.42)	0.05
Controls	1.48±0.14 (1.20–1.76)	1.54±0.21 (1.22–1.86)	0.28

* Previously reported [2]. Data presented as mean ± SD, with range in parentheses.

β₂-Microglobulin

Serum B2M did not change overall following amputation (1.56 ± 0.30 mg/l pre vs. 1.60 ± 0.30 mg/l post amputation, *p* = 0.42), in controls (1.48 ± 0.14 vs. 1.54 ± 0.22 mg/l, *p* = 0.28), or in small (1.44 ± 0.28 vs. 1.48 ± 0.26 mg/l, *p* = 0.74) or medium (1.68 ± 0.36 vs. 1.61 ± 0.28 mg/l, *p* = 0.49) %EBWL amputation subgroups (table 2; fig. 2c). Serum B2M levels appeared to increase in the large amputation subgroup (1.52 ± 0.21 mg/l pre vs. 1.68 ± 0.33 mg/l post amputation, *p* = 0.05; table 2; fig. 2d).

Filtration Marker Combination

One hundred percent (33/33) of amputees had at least one filtration marker that changed <15% after amputation and 88% (29/33) of amputees had a filtration marker that changed <10%. Cystatin C, B2M, and BTP were the most stable filtration marker in 14, 12, and 7 amputees, respectively.

Discussion

This is the first study to investigate changes in the novel filtration markers BTP and B2M following amputation in patients with otherwise normal renal function.

In support of our hypothesis, mean B2M and BTP were unchanged following amputation overall, suggesting that similar to cystatin C, B2M and BTP are superior to SCr for estimating GFR following amputation. Because in all cases at least one filtration marker changed <15% after amputation, it is likely that a GFR estimating equation combining the 3 markers (cystatin C, B2M, and BTP), but not SCr, would be the most valid after amputation. The increased validity of GFR estimation using more than one filtration marker (cystatin C and SCr in the non-amputee population) is already well established [1].

Contrary to our hypothesis, serum concentrations of BTP and B2M were decreased and increased, respectively, following large %EBWL amputation. Because we used stringent selection criteria to include only subjects with normal intrinsic renal function, these differences are likely due to non-GFR determinants. Though BTP is thought to be primarily produced in the heart and brain, it has also been shown to be produced by osteoblasts in vitro and elevated after bone fracture to potentially aid skeletal remodeling [26, 27]. By demonstrating decreased serum BTP levels following large volume skeletal loss, our data suggest that osteoblastic BTP production may make a larger contribution to serum BTP levels than previously thought and provide in vivo evidence linking osteoblastic

Fig. 2. Box and whisker plots, pre- and post-amputation BTP for all amputees (**a**), pre- and post-amputation BTP for large %EBWL amputees (**b**), pre- and post-amputation B2M for all amputees (**c**), pre- and post-amputation B2M for large %EBWL amputees (**d**). Top and bottom of whisker represent maximum and minimum, respectively. Top and bottom of boxes represent 75th and 25th percentile, respectively. Lines in each box represent the median.

BTP production and serum BTP levels. The effect of TBI on choroid plexus BTP production is unknown. No large amputation subject experienced moderate or severe TBI, and TBI incidence did not differ between the medium and large amputation subgroups. Urogenital epithelial cells, vascular endothelial cells, and adipocytes may also produce BTP [20]. These cells were likely lost in higher volume in large %EBWL amputees.

B2M is associated with age, male gender, C-reactive protein (CRP), and white blood cell (WBC) count and is inversely related to systolic BP and cholesterol [28]. Elevated B2M in our large amputee group may be attributable to chronic inflammation. Modestly increased CRP is associated with elevated levels of B2M, but not BTP, SCr, or cystatin C [29]. Amputation is associated with increased pulse wave forms and increased pulse wave forms are associated with inflammation and elevated CRP [30–32]. Increased systemic inflammation could also result from multiple surgeries, procedures, and infections or chronic microtrauma to limb stumps while ambulating on bilateral prostheses. Unfortunately, due to serum volume and financial constraints, we were unable to compare CRP and WBC levels before and after amputation to confirm this theory. In the

future, CRP levels could be obtained in amputees, to further evaluate for increased inflammation in this population.

Our results also broaden our understanding of the elevated cardiovascular morbidity and mortality observed in amputees [6–11]. First, amputees may have undiagnosed CKD due to overestimation of GFR via SCr-based equations [2, 3]. Cardiovascular morbidity and mortality are increased in the setting of CKD [13, 14]. In addition, frequent exposure to nephrotoxins such as nonsteroidal anti-inflammatory drugs and contrast following amputation (table 1) could further exacerbate CKD and subsequent cardiovascular risk. Second, the low BTP levels found in our large amputees could contribute to cardiovascular risk. BTP promotes vasodilatation, induces endothelial atheroprotection, decreases platelet aggregation, stabilizes atherosclerotic plaques, and inhibits adipogenesis [33]. Also, in a study of 3,136 NHANES III participants, subjects with BTP levels in the lowest quartile were more than twice as likely as those in the highest quartile to have metabolic syndrome [34]. Finally, elevated B2M could be a surrogate for increased systemic inflammation as measured by CRP. Higher CRP levels are associated with mortality and cardiac events [35, 36].

More accurate GFR estimation and a better understanding of cardiac risk in traumatic amputees could be applied to the larger population of non-traumatic amputees. An estimated 77% of major limb amputations are due to vascular diseases, and millions of Americans have sustained non-traumatic amputation [4]. GFR estimation is necessary to diagnose and classify CKD as well as to identify the need for nephrology referral, contrast-induced nephropathy prophylaxis, nephrotoxin avoidance, medication dose adjustment, dietary modification, assessment for complications of CKD, and consideration of transplantation [36]. Appreciation of cardiovascular risk is essential for aggressive prophylactic and screening measures to reduce morbidity and mortality [37, 38].

Our study has limitations. mGFR was not obtained. However, since the effect of amputation on GFR is unknown, the absence of pre-amputation mGFR for comparison may limit the utility of post-amputation mGFR. Traumatic amputation is a rare event; so determining mGFRs prior to amputation would be entirely impractical. However, mGFR would be necessary to develop and determine the validity of GFR estimating equations for amputees. A large number of subjects would be required, and our results suggest that BTP and B2M should be considered along with cystatin C for future derivation of GFR estimating equations in amputees. Additionally, non-traumatic amputation should be considered for further study, as this may be a more practical setting for obtaining pre- and post-amputation mGFR.

Additional study is required to confirm our findings, particularly in the large amputee subgroup, where comparison of both pre- and post-amputation BTP and B2M were of borderline significance. Sample size may have been insufficient to detect changes in BTP and B2M levels, particularly within %EBWL subgroups. However, prior analysis of these subjects showed significant decreases in SCr in all amputees and in the medium and large %EBWL subgroups [2]. Analysis is based on 2 serum samples obtained years apart and frozen for years before analysis. However, BTP and B2M serum levels have been shown to be unaffected by freeze-thaw cycles [39], and we used well-characterized methods of BTP and B2M quantification, with small interassay coefficients of variation. Study subjects were free of comorbidities at the time of initial sample collection; we used rigorous criteria to exclude subjects not in a steady state or with undiagnosed CKD, and mean pre- and post-eGFR_{CysC} values were $>110 \text{ ml/min/1.73 m}^2$ in controls, amputees overall, and all subgroups [2].

In conclusion, traumatic amputation often affects young and previously healthy people, and is associated

with increased rates of adverse health outcomes. Accurate GFR estimation is important in this population, and we show for the first time that BTP and B2M are likely to outperform SCr as markers of renal filtration following amputation. Unanticipated changes of uncertain significance in BTP and B2M in the large amputee population suggest that bone loss and chronic inflammation may contribute to the well-documented cardiovascular risk in amputees, and that future study should be undertaken in this group.

Disclaimer

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Disclosure Statement

The authors have no conflicts of interest to declare.

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