

## Length Polymorphisms in Heme Oxygenase-1 and AKI after Cardiac Surgery

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### ABSTRACT

Heme oxygenase-1 (HO-1) catalyzes the degradation of heme, which may be involved in the pathogenesis of AKI. Length polymorphisms in the number of GT dinucleotide repeats in the HO-1 gene (*HMOX1*) promoter inversely associate with *HMOX1* mRNA expression. We analyzed the association between allelic frequencies of GT repeats in the *HMOX1* gene promoter and postoperative AKI in 2377 white patients who underwent cardiac surgery with cardiopulmonary bypass. We categorized patients as having the short allele (S; <27 GT repeats) or long allele (L; ≥27 GT repeats), and defined AKI as an increase in serum creatinine ≥0.3 mg/dl within 48 hours or ≥50% within 5 days, or the need for RRT. Compared with patients with the SS genotype, patients with the LL genotype had 1.58-fold (95% confidence interval, 1.06 to 2.34; *P*=0.02) higher odds of AKI. After adjusting for baseline and operative characteristics, the odds ratio for AKI per L allele was 1.26 (95% confidence interval, 1.05 to 1.50; *P*=0.01). In conclusion, longer GT repeats in the *HMOX1* gene promoter associate with increased risk of AKI after cardiac surgery, consistent with heme toxicity as a pathogenic feature of cardiac surgery-associated AKI, and with HO-1 as a potential therapeutic target.

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Toxicity mediated by elevated levels of free heme and iron is a key contributor to AKI in a variety of animal models,<sup>1–3</sup> and in humans undergoing surgery with cardiopulmonary bypass (CPB).<sup>4,5</sup> Heme oxygenase (HO), the rate-limiting enzyme in the degradation of heme, has important antioxidant, anti-inflammatory, and antiapoptotic functions. HO-1 is the inducible form, whereas HO-2 is constitutively expressed.

HO-1 has a central role in the pathophysiology of AKI in animal models,<sup>6–8</sup> but data on HO-1 in human AKI are sparse. Polymorphisms in the number of guanosine thymidine dinucleotide

[(GT)<sub>n</sub>] repeats in the promoter of the HO-1 gene (*HMOX1*) are inversely associated with *HMOX1* mRNA expression and HO enzyme activity.<sup>9,10</sup> Longer (GT)<sub>n</sub> repeats are associated with increased cardiovascular events and mortality in patients with diabetes mellitus, ESRD, and peripheral artery disease.<sup>9,11,12</sup> However, no study has evaluated the association between the number of (GT)<sub>n</sub> repeats and risk of AKI in a large cohort of patients. We therefore investigated this association in a carefully phenotyped, multicenter cohort of adult patients undergoing cardiac surgery with CPB. We

hypothesized that longer (GT)<sub>n</sub> repeats would be associated with an increased risk of AKI.

We enrolled 2464 white adult patients who underwent cardiac surgery at Brigham and Women's Hospital and the Texas Heart Institute between 2001 and 2014 into a prospective cohort study. After excluding 87 patients based on prespecified criteria (Supplemental Figure 1), the final cohort consisted of 2377 patients. We used DNA fragment analysis to determine the number of (GT)<sub>n</sub> repeats in the *HMOX1* microsatellite promoter. AKI was defined as an absolute increase in serum creatinine (SCr) ≥0.3 mg/dl above the preoperative baseline within the first 48 hours following cardiac surgery, a relative increase in SCr ≥50% above baseline within 5 days following cardiac surgery, or postoperative need for RRT during the primary hospitalization.<sup>13</sup>

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**Genotype Definition and Frequency**

The frequency distribution of (GT)<sub>n</sub> repeats in patients with or without AKI following cardiac surgery is shown in Figure 1A. Consistent with prior studies,<sup>11,14,15</sup> patients with <27 (GT)<sub>n</sub> repeats were classified as having the “short” (S) allele, while patients with ≥27 (GT)<sub>n</sub> repeats

were classified as having the “long” (L) allele. Accordingly, patients were categorized into three mutually exclusive genotypes: SS, SL, or LL.

**Baseline/Operative Characteristics**

Baseline and operative characteristics for the overall cohort, as well as by genotype,

are shown in Table 1. We did not observe any significant differences across genotypes in age, gender, preoperative renal function, comorbidities, or operative characteristics.

**Absolute and Relative Risks of AKI by HMOX1 Genotype**

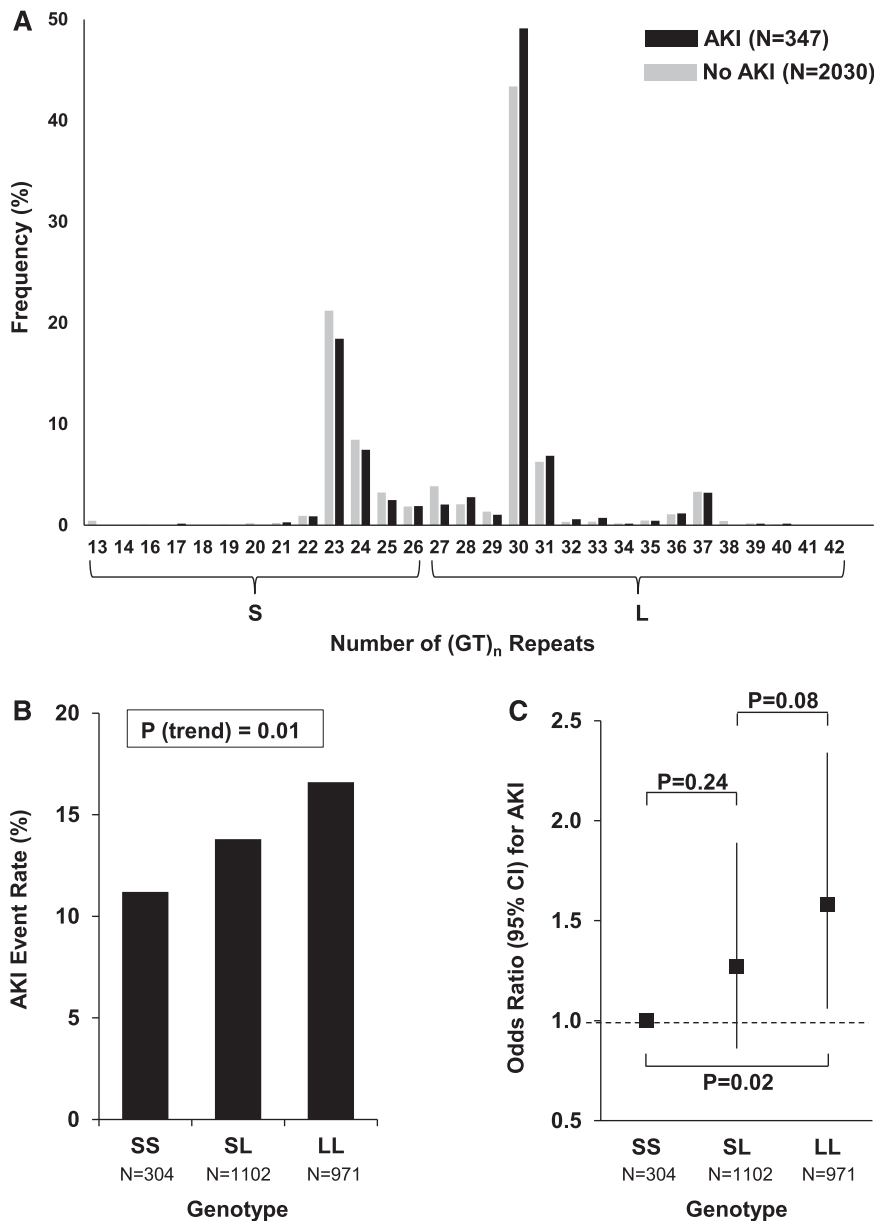
The absolute and relative risks of AKI according to *HMOX1* genotype are shown in Figure 1, B and C. The incidence of AKI among patients with the SS, SL, and LL genotypes was 11.2%, 13.8%, and 16.6%, respectively (*P* for trend = 0.01). Patients with the LL versus the SS genotype had 1.58-fold (95% confidence interval [95% CI], 1.06 to 2.34; *P* = 0.02) higher odds of AKI.

**Univariate and Multivariate Associations between Genotype and AKI**

The univariate and multivariate adjusted odds ratios for risk of AKI according to *HMOX1* genotype, using additive genetic models, are shown in Table 2. Thus, odds ratios represent the incremental risk of AKI for each additional L allele. After adjusting for age, gender, preoperative eGFR, diabetes mellitus, hypertension, prior cardiac surgery, type of procedure (coronary artery bypass graft [CABG] alone, valve alone, or CABG/valve combined), CPB time, number of intraoperative packed red blood cell (pRBC) transfusions, urgent versus non-urgent procedure, and institution, the relative risk of AKI per L allele was 1.26 (95% CI, 1.05 to 1.50; *P* = 0.01). We also investigated three alternative definitions of AKI based on increases in SCr ≥25%, ≥50%, or ≥100% within 5 days following cardiac surgery or need for RRT. In adjusted analyses, we found a significant association between *HMOX1* genotype and an increase in SCr ≥25% within 5 days or need for RRT. We found similar magnitudes of association between *HMOX1* genotype and larger increases in SCr (*i.e.*, ≥50% and ≥100%), with similar odds ratios, but these findings were not statistically significant (Table 2).

**Sensitivity Analysis**

We conducted a sensitivity analysis in which the S allele was defined as <25



**Figure 1.** Frequency distribution of (GT)<sub>n</sub> repeats and risk of AKI by *HMOX1* genotype. (A) The number of (GT)<sub>n</sub> repeats ranged from 13 to 42 and showed a bimodal distribution, with one peak located at 23 and the other located at 30 repeats. The overall frequency distribution of (GT)<sub>n</sub> repeats was not significantly different between patients with versus without AKI (*X*<sup>2</sup> test, *P* = 0.09). However, longer (GT)<sub>n</sub> repeats were associated with an increased risk of AKI (Mantel–Haenszel test for trend, *P* = 0.01). (B) Absolute risk of AKI by *HMOX1* genotype. (C) Relative risk of AKI by *HMOX1* genotype.

**Table 1.** Baseline/operative characteristics

Characteristic	All (n=2377)	HMOX1 Genotype			P Value <sup>a</sup>
		SS (n=304)	SL (n=1102)	LL (n=971)	
Demographics					
Age, yr	65 [58–74]	64 [57–73]	65 [58–74]	66 [58–74]	0.49
Female	529 (22)	69 (23)	231 (21)	229 (24)	0.35
Preoperative renal function					
SCr, mg/dl	1.0 [0.9–1.2]	1.0 [0.9–1.2]	1.0 [0.9–1.2]	1.0 [0.9–1.2]	0.43
eGFR, ml/min per 1.73 m <sup>2</sup>	72 [58–87]	74 [59–87]	72 [58–86]	73 [57–87]	0.56
eGFR<60 ml/min per 1.73 m <sup>2</sup>	692 (29)	83 (27)	315 (29)	294 (30)	0.53
eGFR=30–59 ml/min per 1.73 m <sup>2</sup>	652 (27)	79 (26)	298 (27)	275 (28)	0.67
eGFR=15–29 ml/min per 1.73 m <sup>2</sup>	40 (2)	4 (1)	17 (2)	19 (2)	0.66
Comorbidities					
Hypertension	1764 (74)	217 (71)	834 (76)	713 (73)	0.21
Diabetes mellitus	739 (31)	88 (29)	341 (31)	310 (32)	0.61
Chronic lung disease	260 (11)	35 (12)	130 (12)	95 (10)	0.30
Atrial fibrillation/flutter	193 (8)	28 (9)	91 (8)	74 (8)	0.65
Operative characteristics					
Type of procedure					0.77
CABG alone	1822 (77)	241 (79)	845 (77)	736 (76)	
Valve alone	323 (14)	38 (13)	151 (14)	134 (14)	
Combined CABG and valve	232 (10)	25 (8)	106 (10)	101 (10)	
Urgent procedure	1138 (48)	141 (46)	522 (47)	475 (49)	0.65
Previous cardiac surgery	109 (5)	14 (5)	50 (5)	45 (5)	0.99
CPB time, min	103 [75–137]	102 [75–129]	103 [75–137]	104 [75–138]	0.54
Crossclamp time, min	76 [55–101]	76 [58–98]	77 [55–102]	76 [56–103]	0.94
Intraoperative pRBCs, no. of units	0 [0–1]	0 [0–1]	0 [0–0]	0 [0–1]	0.66
Intraoperative cell saver, ml	3 [2–197]	3 [2–47]	3 [2–130]	3 [2–220]	0.59

Data are presented as n (%) or median [interquartile range, 25th–75th percentile]. The S allele represents a short (<27) number of repeats, and the L allele represents a long (≥27) number of (GT)<sub>n</sub> repeats in the *HMOX1* gene promoter. Shorter repeats are associated with greater *HMOX1* mRNA expression and enzyme activity. eGFR was determined using the Chronic Kidney Disease Epidemiology Collaboration equation.<sup>32</sup>

<sup>a</sup>P values for comparisons among the three groups were calculated using Kruskal–Wallis and X<sup>2</sup> tests for continuous and categorical variables, respectively.

repeats and the L allele was defined as ≥25 repeats.<sup>16,17</sup> In analyses adjusted for the same covariates as above, we found a significant association between *HMOX1* genotype and risk of AKI, with an odds ratio of 1.25 (95% CI, 1.03 to 1.50; *P*=0.02) per L allele. Similar findings were observed using 29 repeats as

the cutoff, with an odds ratio of 1.29 (95% CI, 1.09 to 1.54; *P*=0.004) per L allele.

### Subgroup Analyses

To investigate the possibility of effect modification due to differences in baseline or operative characteristics, we compared the association between *HMOX1* genotype

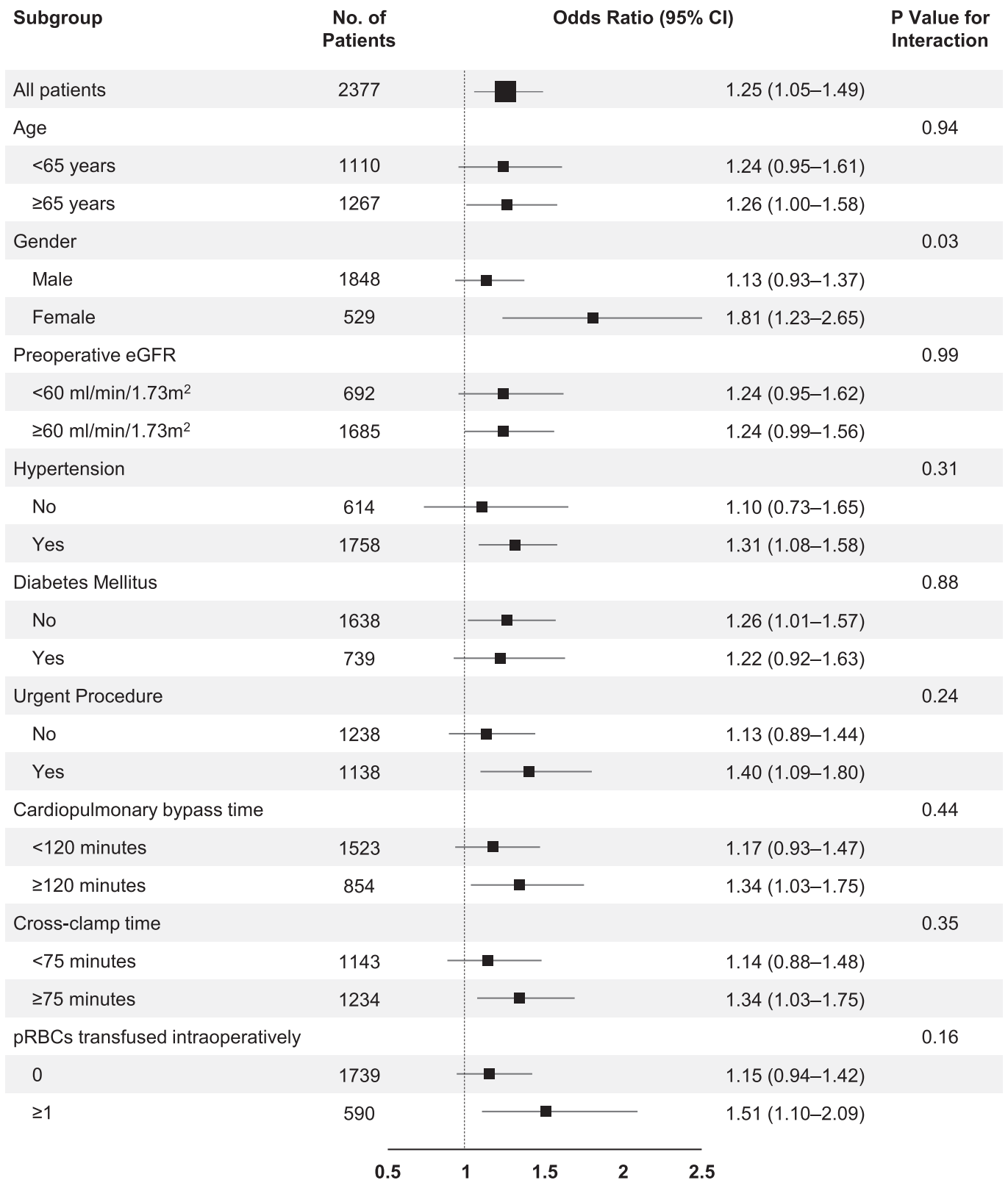
(using 27 repeats as the cutoff) and risk of AKI in subgroup analyses (Figure 2). We found a significantly stronger association between *HMOX1* genotype and risk of AKI in females compared with males, and a non-significant trend toward a stronger association in patients who were transfused ≥1 versus 0 units of pRBCs intraoperatively.

**Table 2.** Risk of AKI following cardiac surgery based on *HMOX1* genotype

End Point	Event Rates, n (%)				Unadjusted		Adjusted	
	All (n=2377)	SS (n=304)	SL (n=1102)	LL (n=971)	Odds Ratio <sup>a</sup> (95% CI)	P Value	Odds Ratio <sup>a</sup> (95% CI)	P Value
Primary end point								
AKI	347 (14.6)	34 (11.2)	152 (13.8)	161 (16.6)	1.25 (1.05 to 1.49)	0.01	1.26 (1.05 to 1.50)	0.01
Secondary end points								
Increase in SCr ≥25% in 5 d or RRT	492 (20.7)	51 (16.8)	222 (20.2)	219 (22.6)	1.19 (1.02 to 1.38)	0.03	1.18 (1.02 to 1.38)	0.03
Increase in SCr ≥50% in 5 d or RRT	171 (7.2)	19 (6.3)	72 (6.5)	80 (8.2)	1.20 (0.95 to 1.52)	0.13	1.20 (0.94 to 1.53)	0.14
Increase in SCr ≥100% in 5 d or RRT	48 (2.0)	5 (1.6)	21 (1.9)	22 (2.3)	1.18 (0.77 to 1.83)	0.45	1.20 (0.76 to 1.89)	0.43

AKI was defined as an absolute increase in SCr ≥0.3 mg/dl above baseline within the first 48 hours following cardiac surgery, a relative increase in SCr ≥50% above baseline within 5 days following cardiac surgery, or postoperative need for RRT.<sup>13</sup>

<sup>a</sup>Additive genetic models for each L allele. Adjusted models include age, gender, preoperative eGFR, diabetes mellitus, hypertension, prior cardiac surgery, type of procedure (CABG alone, valve alone, or CABG/valve combined), CPB time, number of intraoperative pRBC transfusions, urgent procedure, and institution.



**Figure 2.** Forest plot showing risk of AKI following cardiac surgery based on *HMOX1* genotype-subgroup analyses. Odds ratios were computed using additive genetic models, and therefore represent the incremental risk of AKI with each additional L allele.

### Plasma Free Hemoglobin and Other Plasma Markers

Finally, to explore possible mechanisms responsible for the association between *HMOX1* genotype and risk of AKI following CPB, we measured plasma levels of free hemoglobin, ferritin, transferrin saturation, and catalytic iron longitudinally in a subgroup of 192 patients at high risk of postoperative AKI. At each of the three postoperative time points, we found a trend toward higher plasma free hemoglobin levels with each additional L allele (Supplemental Figure 2). Postoperative levels of ferritin, transferrin saturation, and catalytic iron did not differ by genotype (data not shown).

Thus, in this prospective cohort study, we report that patients with longer (GT)<sub>n</sub> repeats in the *HMOX1* gene promoter had an increased risk of AKI following cardiac surgery. The risk of AKI was increased 1.25-fold (95% CI, 1.05 to 1.49; *P*=0.02) for each additional L allele, and individuals with the LL genotype had a 1.58-fold (95% CI, 1.06 to 2.34; *P*=0.02) increased risk of AKI compared with patients with the SS genotype. Further, these associations remained after adjusting for covariates included in multivariable models.

The potent renoprotective effects of HO-1 have been well established in a wide variety of experimental settings, including models of renal injury induced by ischemia-reperfusion<sup>18</sup> and LPS,<sup>8</sup> and are reviewed in detail elsewhere.<sup>6</sup> Proposed mechanisms include: inhibition of autophagy<sup>19</sup>; anticoagulant effects<sup>20</sup>; increased synthesis of ferritin,<sup>21</sup> which sequesters free iron; and production of carbon monoxide and biliverdin, which have antioxidant and renoprotective properties.<sup>22,23</sup> Additionally, a recent study demonstrated the importance of myeloid-specific *HMOX1* expression in the regulation of immune cell trafficking after ischemia-reperfusion injury.<sup>24</sup>

In contrast to these comprehensive studies in animal models of renal injury, data on HO-1 in human AKI are sparse. In several small studies, HO-1 protein levels were shown to be elevated in plasma samples taken from patients with AKI.<sup>25,26</sup> Although intriguing, plasma HO-1 levels are difficult to interpret in

this context due to confounding by severity of illness. We therefore used a Mendelian randomization approach, since it limits bias. This approach has been used to study the association between *HMOX1* genotype and a variety of outcomes, including cardiovascular events and mortality,<sup>11</sup> incident CKD,<sup>14</sup> and chronic allograft nephropathy,<sup>27</sup> but the association with AKI was evaluated in only a single small study. Askenazi *et al.* found no association between the number of (GT)<sub>n</sub> repeats in the HO-1 gene promoter and risk of AKI in 117 premature infants.<sup>28</sup> However, with only 34 AKI events, the investigators likely did not have sufficient power to detect an association.

We conducted several subgroup analyses and found a significantly stronger association between *HMOX1* genotype and risk of AKI in females compared with males, and a trend toward a stronger association in patients transfused  $\geq 1$  versus 0 units of pRBCs intraoperatively. The association with transfusions is particularly interesting, since pRBCs undergo hemolysis over time, resulting in the release of free hemoglobin.<sup>29</sup> The association with gender has not been reported in prior human studies to the best of our knowledge; however, Pósa *et al.* found greater HO-1 expression and activity in female compared with male rat aorta and left ventricle. They concluded that gender-related differences in HO-1 activity may play a role in the sexual dimorphism of susceptibility to cardiovascular disease.<sup>30</sup>

The mechanisms responsible for our observation of greater risk of AKI in patients with longer (GT)<sub>n</sub> repeats could not be determined in this observational study. However, a highly plausible mechanism involves decreased *HMOX1* expression in patients with longer (GT)<sub>n</sub> repeats,<sup>9,10</sup> resulting in diminished capacity to catabolize toxic free heme. While the mechanisms of cardiac surgery-associated AKI are complex and multifactorial, hemolysis is likely a major contributor.<sup>4,5</sup> CPB induces hemolysis by exposure of red blood cells to nonphysiologic surfaces, shear stress generated by pumps and suction systems, and frequent transfusion of pRBCs.<sup>29,31</sup> HO-1 mitigates the effects of hemolysis by

catabolizing heme into carbon monoxide, biliverdin, and iron. The latter becomes sequestered by ferritin, which is upregulated by HO-1. Thus, it is likely that patients with decreased *HMOX1* expression caused by longer (GT)<sub>n</sub> repeats have an increased susceptibility to heme-related, cardiac surgery-associated AKI.

We acknowledge several limitations of our study, including the observational design. Although we measured plasma free hemoglobin levels longitudinally in a subcohort of patients and documented higher levels in patients with longer (GT)<sub>n</sub> repeats, we did not measure other potentially relevant plasma markers, such as HO-1 protein levels or bilirubin levels. However, the inverse association between the number of (GT)<sub>n</sub> repeats in the *HMOX1* gene promoter and *HMOX1* mRNA expression has been well documented in prior studies.<sup>9,10</sup> Finally, we did not have data on long-term renal outcomes, such as incidence or progression of CKD.

In conclusion, we found that longer (GT)<sub>n</sub> repeats in the *HMOX1* gene promoter, which are known to affect *HMOX1* expression, were associated with an increased risk of AKI following cardiac surgery. Future studies are indicated to explore pharmacologic targeting of *HMOX1* expression as a strategy for AKI prevention in humans.

## CONCISE METHODS

### Study Design

We enrolled patients undergoing cardiac surgery into a prospective cohort study at Brigham and Women's Hospital (Boston, MA) and the Texas Heart Institute (Houston, TX) between 2001 and 2014. All patients provided written informed consent, including consent specifically for genetic studies, and all protocols were approved by the respective institutional review boards.

### Study Patients

Subjects with a preoperative hematocrit <25% or those who received transfusion of leukocyte-rich blood products within 30 days before surgery were not enrolled. We restricted our analyses to subjects of white ancestry to avoid the potential influence of

population stratification on the observed associations. Additionally, we excluded the following groups of patients: cardiac surgery without CPB; emergency surgeries; missing preoperative SCr data; missing postoperative SCr data (patients were required to have at least one SCr value within 5 days following cardiac surgery to be included); preoperative eGFR <15 ml/min per 1.73 m<sup>2</sup>; history of renal transplantation; and ESRD (Supplemental Figure 1).

### Data and Sample Collection

Data were collected for each enrolled subject during their hospitalization using detailed case report forms that included: (1) preoperative demographic characteristics and comorbidities; (2) surgical characteristics; and (3) postoperative data, including daily SCr values for the first 5 days after surgery. Data were subjected to automated range and logic checking, as well as a manual audit of records to ensure data quality.

Plasma was collected preoperatively and on postoperative days 1 through 5, and stored at -80°C within 2 hours of collection. DNA was extracted from white blood cells using standard protocols.

### HMOX1 Genotyping

We used DNA fragment analysis to determine the number of (GT)<sub>n</sub> repeats in the *HMOX1* microsatellite promoter. The *HMOX1* locus containing the GT repeat was amplified using PCR with fluorescently labeled primers. All amplicons were separated by size using capillary electrophoresis on an ABI3730 sequencer. Data analysis was performed with GeneMapper 5 software (Thermo Fisher Scientific, Vernon Hills, IL), including automatic sizing and allele calling. Two individuals were Sanger sequenced and the number of repeats was counted. This information was used to convert amplicon lengths to repeat numbers. Consistent with prior studies, we used 27 repeats as the cutoff for genotype classification.<sup>11,14,15</sup> Patients with <27 (GT)<sub>n</sub> repeats were classified as having the S allele, while patients with ≥27 (GT)<sub>n</sub> repeats were classified as having the L allele.

### End Points

The prespecified primary end point was postoperative AKI, defined as an absolute increase

in SCr ≥0.3 mg/dl above baseline within the first 48 hours following cardiac surgery, a relative increase in SCr ≥50% above baseline within 5 days following cardiac surgery, or postoperative need for RRT. These criteria are identical to those established by the Kidney Disease Improving Global Outcomes Work Group,<sup>13</sup> with the exception of 5 days instead of 7 days. Secondary end points included the following alternative definitions of AKI: increases in SCr ≥25%, ≥50%, or ≥100% above baseline within 5 days following cardiac surgery or need for RRT.

### Subgroup Analyses

We evaluated the association between *HMOX1* genotype and risk of AKI in the following subgroups: age (<65 versus ≥65 years); gender; preoperative eGFR (<60 versus ≥60 ml/min per 1.73 m<sup>2</sup>); hypertension; diabetes mellitus; urgent procedure; CPB time (<120 versus ≥120 minutes); crossclamp time (<75 versus ≥75 minutes); and pRBCs transfused intraoperatively (0 versus ≥1 unit).

### Sensitivity Analyses

We conducted two sensitivity analyses in which the S allele was defined as <25 repeats and the L allele was defined as ≥25 repeats,<sup>16,17</sup> and in which the S allele was defined as <29 repeats and the L allele was defined as ≥29 repeats.

### Plasma Measurements

We measured plasma levels of free hemoglobin, ferritin, transferrin saturation, and catalytic iron in a subcohort of 192 patients at high risk of postoperative AKI. Patients were considered to be at high risk of AKI if they had a baseline eGFR ≤30 ml/min per 1.73 m<sup>2</sup> or any two of the following: baseline eGFR = 31–60 ml/min per 1.73 m<sup>2</sup>, diabetes mellitus, left ventricular ejection fraction ≤40%, previous cardiac surgery, combined CABG/valve procedure, urgent procedure, and preoperative intraaortic balloon pump.<sup>4</sup> The above measurements were performed on plasma samples collected at four time points: preoperatively, at the end of CPB, and on postoperative days 1 and 3. The details of these measurements are reported elsewhere.<sup>4</sup> In brief, plasma free hemoglobin levels were measured using an ELISA kit (catalog no. MBS 564144; MyBioSource, San Diego, CA). Ferritin levels were measured using a

two site immunoenzymometric assay kit (Tosoh Corporation, Japan). Transferrin saturation (%) was calculated as the ratio of total iron/total iron binding capacity, multiplied by 100. Catalytic iron was measured using the modified bleomycin assay. The interassay coefficient of variation for all assays, estimated using blinded split samples from study patients, was <10%.

### Statistical Analyses

Statistical analyses were performed with SAS Version 9.4 (SAS Institute Inc., Cary, NC). Data are reported as median and interquartile range (25th–75th percentiles). Baseline/operative characteristics were compared in patients across *HMOX1* genotypes using Kruskal–Wallis and  $\chi^2$  tests for continuous and categorical variables, respectively. The Mantel–Haenszel test for trend was used to assess the association between longer (GT)<sub>n</sub> repeats and risk of AKI. The Cochran–Armitage test for trend was used to assess the unadjusted association between *HMOX1* genotype and incidence of AKI.

Logistic regression was used to assess the association between *HMOX1* genotype and AKI, using additive genetic models. Multivariate models were adjusted for age, gender, preoperative eGFR, diabetes mellitus, hypertension, prior cardiac surgery, type of procedure (CABG alone, valve alone, or CABG/valve combined), CPB time, number of intraoperative pRBC transfusions, urgent versus nonurgent procedure, and institution. Comparison of plasma free hemoglobin levels at individual time points across *HMOX1* genotypes was assessed using Spearman rank correlation coefficient. All comparisons are two-tailed, with  $P<0.05$  considered significant.

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## DISCLOSURES

None.

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