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The effect of dietary modulation of sulfur amino acids on cystathionine β synthase–deficient mice

Warren D. Kruger and Sapna Gupta

Cancer Biology Program, Fox Chase Cancer Center, Philadelphia, Pennsylvania

Abstract

Cystathionine β synthase (CBS) is a key enzyme in the methionine and cysteine metabolic pathway, acting as a metabolic gatekeeper to regulate the flow of fixed sulfur from methionine to cysteine. Mutations in the CBS gene cause clinical CBS deficiency, a disease characterized by elevated plasma total homocysteine (tHcy) and methionine and decreased plasma cysteine. The treatment goal for CBS-deficient patients is to normalize the metabolic values of these three metabolites using a combination of vitamin therapy and dietary manipulation. To better understand the effectiveness of nutritional treatment strategies, we have performed a series of long-term dietary manipulation studies using our previously developed *Tg-I278T Cbs*^{−/−} mouse model of CBS deficiency and sibling *Tg-I278T Cbs*^{+/-} controls. *Tg-I278T Cbs*^{−/−} mice have undetectable levels of CBS activity, extremely elevated plasma tHcy, modestly elevated plasma methionine, and low plasma cysteine. They exhibit several easily assayable phenotypes, including osteoporosis, loss of fat mass, reduced life span, and facial alopecia. The diets used in these studies differed in the amounts of sulfur amino acids or sulfur amino acid precursors. In this review, we will discuss our findings and their relevance to CBS deficiency and the concept of gene–diet interaction.

Keywords

methionine; homocysteine; alopecia; fat mass; metabolism

Introduction to sulfur amino acid metabolism

Methionine is an essential dietary amino acid in mammals because mammals lack the enzymes necessary to fix inorganic sulfur to carbon. Cysteine, the other sulfur-containing amino acid, is not essential because it can be derived from methionine via the transsulfuration pathway (Fig. 1). Methionine, besides being used in protein synthesis, provides one-carbon units for a variety of methylation reactions via its conversion to *S*-adenosylmethionine. Once the methyl group is donated, *S*-adenosylhomocysteine is formed, which is in turn hydrolyzed to homocysteine. Homocysteine lies at the key regulatory point in the pathway. If homocysteine is remethylated back to methionine, using methyl groups

Address for correspondence: Warren D. Kruger, PhD, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111. warren.kruger@fccc.edu.

Conflicts of interest

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supplied by either methyl-tetrahydrofolate or betaine, you have a closed cycle, known as the methionine cycle. Alternatively, homocysteine can be condensed with serine to form cystathionine by the enzyme cystathionine β synthase (CBS). This reaction is irreversible, and thus leads to loss of fixed sulfur from the methionine cycle.

The cystathionine produced by the CBS reaction is cleaved by the enzyme cystathionine γ lyase to form α -ketobutyrate and the amino acid cysteine. The cysteine produced in the cell can either be used for protein synthesis or be converted to other sulfur-containing small molecules, such as glutathione or taurine. The process by which homocysteine is converted to cysteine is called transsulfuration. In cell culture models, it is estimated that 50% of all methionine taken up is converted to cysteine by transsulfuration,¹ although in human whole-body studies the amount is only 20%.² The fact that a significant portion of methionine is normally utilized for cysteine formation is responsible for the so-called “methionine-sparing” effect of cysteine.³ This simply refers to the fact that, under conditions of cysteine availability, less methionine is needed than under conditions of cysteine starvation.

CBS deficiency

Cystathionine β synthase deficiency is a recessive inborn error of metabolism caused by mutations in *CBS*.⁴ It is the most common inborn error of sulfur metabolism and characterized by very high levels of plasma total homocysteine (tHcy), plasma methionine, and low levels of total cysteine (tCys). Total homocysteine and tCys refer to the sum total of all forms of homocysteine or cysteine found in blood, including both the free reduced form and oxidized forms that are cross-linked to other thiol-containing compounds.⁵ It should be noted that protein-bound homocysteine and cysteine make up the vast majority of both compounds in blood. CBS patients suffer from numerous complications, including thrombosis, ectopia lentis, osteoporosis, brittle and thin skin, fine fair hair, fatty liver, mental retardation, and psychiatric disturbances.⁶ It is estimated to occur in about 1/100,000 births in the United States, although rates vary significantly in different countries.⁷ The major cause of mortality and morbidity in these patients is thrombosis. The treatment goal for CBS-deficient patients is to decrease tHcy levels, as lower tHcy levels are associated with improved outcomes.⁸ The first treatment strategy described was to restrict the level of methionine in the diet and supplement with cysteine.^{9–11} Soon after, it was discovered that a subset of patients responded to pyridoxine supplementation, which may be related to the CBS enzyme's use of pyridoxal phosphate as a co-factor.¹² Later, betaine supplementation was found to be effective in lowering patients' tHcy.¹³ Betaine is a co-substrate of the enzyme BHMT, which catalyzes the formation of methionine from homocysteine in the liver. Thus, increased betaine is thought to lower homocysteine levels by decreasing the homocysteine pool and increasing the methionine pool. Because the number of CBS-deficient patients is quite small, none of these different treatment strategies has ever been tested directly in a controlled clinical environment, and thus the relative effectiveness of each of these strategies in isolation is unknown. It is also important to note that dietary compliance for methionine is the most difficult clinical aspect of treating CBS-deficient patients.¹⁴

Mouse models of CBS deficiency

To understand the pathogenic mechanism of elevated tHcy, Watanabe and colleagues¹⁵ created a mouse that contained a CBS knockout allele (*Cbs*^{-/-}). It was found that *Cbs*^{-/-} homozygotes were born at the expected Mendelian frequency but had a neonatal lethal phenotype in which ~ 90% of the animals die between 3 and 6 weeks of age owing to liver dysfunction. In order to circumvent this problem, our lab created a transgenic mouse (*Tg-hCBS*) in which the human CBS cDNA is under control of the zinc-inducible metallothionein promoter.¹⁶ The construct contains the core mouse *MT-1* promoter flanked by two 10-kb locus-control regions. As initially shown by Palmiter and confirmed by our own group, these locus-control regions are extremely effective in abrogating variable expression of the transgene due to local insertion site effects.^{16,17} By crossing *Tg-hCBS* mice with *Cbs*^{+/-} mice, *Tg-hCBS Cbs*^{+/-} animals were generated that could then be intercrossed on zinc water to generate *Tg-hCBS Cbs*^{-/-} mice. These mice were viable and, at weaning, were alive in the expected Mendelian proportions.¹⁶ Upon weaning, zinc was removed and the mice could be aged with no obvious phenotypes, despite having tHcy levels averaging 169 µM.¹⁸ Examination of liver and serum tHcy levels showed that the wild-type human CBS enzyme was enzymatically active and its expression was appropriately modulated by zinc.

We next created a mouse model that contained a transgene that expressed a human CBS protein containing an isoleucine-to-valine substitution at position 278 (*Tg-I278T*). This mutation is the most common allele found in CBS-deficient patients.¹⁹ When treated with zinc, these animals tend to have reduced steady-state levels of CBS protein compared to *Tg-hCBS* animals, but the protein that is produced has only about 3% of the specific activity of wild-type CBS.²⁰ Surprisingly, *Tg-I278T Cbs*^{-/-} animals also survive the neonatal period even though they have tHcy levels ~290 µM.²⁰ These findings suggest that CBS protein, as opposed to enzyme activity, is important for proper neonatal liver development and that the I278T mutant protein can still work in this regard.

Adult *Tg-I278T Cbs*^{-/-} mice have serum tHcy about 50-fold higher, serum methionine 1.8-fold higher, and a 2-fold decrease in serum tCys compared to sibling *Tg-I278T Cbs*^{+/-} control mice. Unlike the *Tg-hCBS Cbs*^{-/-} mice, they have a variety of well-characterized age-related phenotypes, including osteoporosis, decreased weight gain, low percent body fat, facial alopecia, and reduction of liver SCD-1 protein levels^{18,21} (Fig. 2). All of these phenotypes get more pronounced as the animals become older. The fact that these phenotypes are not observed in *Tg-hCBS Cbs*^{-/-} mice suggests that there may be a distinct tHcy threshold at which tHcy becomes toxic. Consistent with this idea, data from Irish CBS patients shows that treated patients have an 18-fold decrease in thrombosis rates compared to untreated patients, even though the mean treated tHcy level was 108 µM.²²

How do the phenotypes of *Tg-I278T Cbs*^{-/-} mice compare to human patients with CBS deficiency? The metabolic sequelae of these mice is similar to human patients, with extremely elevated tHcy, decreased tCys, and increased methionine. However, the methionine increases observed in human patients are much greater than those typically observed in *Tg-I278T Cbs*^{-/-} mice.^{18,23} However, in young *Cbs*^{-/-} mice (before weaning),

methionine levels are much higher than in adult mice and more closely mimic the human situation (W.K., unpublished data). With regard to osteoporosis and weight loss, both of these phenotypes have been reported in human CBS-deficient patients. Interestingly, CBS-deficient patients were noted to resemble Marfan's syndrome patients with dolichostenomelia and other skeletal abnormalities, including osteoporosis.^{24,25} Furthermore, examination of BMI in untreated patients (see Table 1 in Ref. 24) shows that they tend to be low relative to age-matched population controls.

However, the main cause of morbidity in CBS-deficient patients is thrombosis, and we have observed no spontaneous thrombotic events in *Tg-I278T Cbs^{-/-}* mice, despite generating around 2000 animals. In addition, *Tg-I278T Cbs^{-/-}* mice do not display increased susceptibility to arterial or venous thrombosis using photochemical injury to the carotid or chemical injury to either the carotid or mesenteric arterioles.²⁶ However, *Tg-I278T Cbs^{-/-}* mice display evidence of endothelial dysfunction, and endothelial cells are known to play key roles in the thrombotic process *in vivo*.²⁷ The lack of a thrombotic phenotype in the mice may simply reflect inherent differences in human and mouse biology, as it is known that mouse and human platelets differ in specific aspects of thrombin signaling.²⁸

The easily quantifiable age-related phenotypes in the *Tg-I278T Cbs^{-/-}* mice make it an attractive model for studying various dietary strategies to ameliorate the effects of CBS deficiency. In addition, by comparing the effects of dietary modulation in both *Cbs^{-/-}* and *Cbs^{+/-}* mice, we can also explore gene–diet interactions. These studies will be explored in the next section.

Dietary interventions: overview and experimental design

The overview of the experimental set-up for our dietary intervention studies is shown in Figure 3. To generate the animals, *Tg-I278T Cbs^{-/-}* males are bred to *Tg-I278T Cbs^{+/-}* females on zinc water, resulting in half the animals being *Cbs^{-/-}* and half being *Cbs^{+/-}*. Because the *Tg-I278T* mice are not 100% C57BL6,¹⁶ the use of sibling controls is essential to determine the effect of the CBS genotype. When the pups are 1 month old, they are put into new cages and switched over to non-zinc water and given either control or experimental diet. The mice are then aged until they are 240 days old. During the aging process, the mice are weighed monthly and photographed to assess the development of the alopecia phenotype. When the mice reach 240 days of age, the animals are euthanized, serum and liver are collected, and the carcasses are subjected to dual-energy X-ray absorptiometry (DEXA) scanning to assess fat mass, lean mass, and bone mineral density. The serum is used to determine tHcy, tCys, and methionine levels, while liver extracts are used to measure the levels of SCD-1.

Because the dietary intervention studies were not performed at the same time, we always include some additional animals on control diet when doing a new dietary intervention. These so-called “controls for controls” allow us to be confident that the DEXA and weight measurements are stable over time, thereby allowing comparison between all experiments.

The phenotypes of *Cbs^{-/-}* and *Cbs^{+/-}* mice on four different diets were evaluated. The diets were control regular diet (RD), *N*-acetyl L-cysteine (NAC)–supplemented, methionine–

restricted (MRD), and betaine-supplemented (BSD) diet. Below we will describe each intervention in detail.

N-acetylcysteine supplementation

Adult *Tg-I278T Cbs*^{-/-} mice have a 64% decrease in serum tCys and a 34% decrease in liver glutathione levels compared to sibling heterozygous controls (Fig. 4). This is particularly interesting since a variety of experimental and epidemiological studies show a connection between cysteine levels and obesity,²⁹ leading to the hypothesis that the loss of fat mass observed in *Tg-I278T Cbs*^{-/-} mice might be related to the low tCys levels. Consistent with this idea is the finding that mice lacking the glutamate–cysteine ligase modifier gene (*GCLM*) show decreased fat mass and are resistant to weight gain on a high-fat diet.³⁰ In addition, since glutathione is a key player in cellular redox defenses, it might also explain the increasing severity of the phenotypes with age.

To test this, drinking water was supplemented with 40 mM NAC.²¹ NAC is similar to cysteine, but the presence of the acetyl moiety makes it less susceptible to oxidation and more soluble in water.³¹ In addition, it has been widely used as a human supplement and has been used in a variety of clinical trials to treat conditions such as bronchitis, cystic fibrosis, and HIV infections.^{31–33} NAC treatment raised serum tCys from 102 to 153 μ M, although this was still less than the 281 μ M observed in heterozygous control animals (Fig. 4C). With respect to liver glutathione (GSH), NAC-treated *Tg-I278T Cbs*^{-/-} mice had levels that were indistinguishable from controls (see Ref. 20). NAC treatment did not affect either tCys or liver GSH in control animals.²¹

Despite the improvement in both tCys and liver GSH, there were no other phenotypic improvements. No significant differences were observed in the weight gain (Fig. 5A), fat mass (Fig. 5B), osteoporosis (Fig. 5C), or alopecia (Fig. 6) phenotypes in *Tg-I278T Cbs*^{-/-} mice.²¹ In addition, NAC did not increase the expression of SCD-1 in the liver (see Fig. 4 in Ref. 20). In fact, NAC had a negative effect on the growth of both *Tg-I278T Cbs*^{-/-} and *Tg-I278T Cbs*^{+/-} mice over the course of the study, but the effect was more severe in the heterozygous mice (Fig. 5A). In control animals, we observed large decreases in both lean and fat mass at the end of the study (Fig. 5B and Ref. 20). The reason for the negative effects of NAC on growth are unknown, but there are some reports linking NAC in drinking water and weight loss.^{34,35} One important difference between NAC and cysteine is that NAC is a potent antioxidant while cysteine is not.

In summary, the NAC studies suggest that low cysteine and glutathione levels are not directly related to the major phenotypes in *Tg-I278T Cbs*^{-/-} mice and hint that cysteine supplementation may not be beneficial in human patients with CBS deficiency. However, because tCys levels were not fully corrected by the intervention, we cannot entirely exclude the possibility that tCys may not have been elevated sufficiently to reverse the phenotypes.

Low-methionine intervention

We next examined the effect of a low-methionine diet on our mice.³⁶ Because all homocysteine is ultimately derived from dietary methionine, lowering dietary methionine

should reduce both serum tHcy and methionine levels. For these studies, we created an MRD that contains only 8.5% of the methionine contained in an RD.

This diet was very effective in reducing both tHcy and methionine levels. In *Tg-I278T Cbs^{-/-}* mice, tHcy decreased from 357 μ M on RD to 81 μ M on MRD, and methionine plummeted from 142 μ M to 15 μ M. Unexpectedly, the diet also increased tCys levels from 102 μ M to 238 μ M, and was even more effective than NAC in this regard. The possible explanation for this result is that methionine restriction is preventing the loss of cysteine by blocking the formation of homocysteine–cysteine disulfide. Since plasma homocysteine and homocysteine–cysteine mixed disulfide are reabsorbed in the kidney via the luminal cystine/basic amino acid transporter, increased urinary homocysteine and mixed disulfide excretion occur when the capacity of the transporter is exceeded.³⁷ We hypothesize that, in CBS deficiency, a significant amount of cysteine is lost due to this mechanism. Consistent with this idea is the observation that CBS-deficient patients have been shown to excrete large amounts of homocysteine–cysteine disulfide in the urine.³⁸

Unlike NAC, MRD was extremely effective at correcting all the assessed phenotypes. We found that both male and female *Tg-I278T Cbs^{-/-}* mice grew significantly faster when on MRD (Fig. 5A). In fact, by the end of the 240-day experiment, *Tg-I278T Cbs^{-/-}* mice on MRD weighed the same as control *Tg-I278T Cbs^{+/-}* mice on RD. The fat mass (Fig. 5B), osteoporosis (Fig. 5C), and hair loss phenotypes (Fig. 6) were all fully corrected as well. We also observed a large increase in the levels of liver SCD-1. However, despite these improvements, the mean tHcy levels of *Tg-I278T Cbs^{-/-}* mice on MRD was still more than 10-fold elevated relative to control mice. This finding is consistent with our previous observation that *Tg-hCBS Cbs^{-/-}* mice, which have mean tHcy levels that are 100–200 μ M less than those of *Tg-I278T Cbs^{-/-}* mice, do not show any of the major phenotypes.¹⁸ This suggests that, in mice, there is a threshold level that tHcy must cross for phenotypes to become evident.

A particularly intriguing observation was that *Tg-I278T Cbs^{-/-}* mice on MRD were actually significantly heavier than sibling *Tg-I278T Cbs^{+/-}* mice on MRD (Fig. 5A). A possible explanation for this finding is that there is less “methionine wastage” due to an inactive transsulfuration pathway. Because of the cyclical nature of methionine metabolism (Fig. 1), methionine can only be lost for two reasons: incorporation into protein or conversion to cysteine by the action of CBS. For mice on MRD, methionine for protein synthesis is probably rate limiting for growth, so any methionine converted to cysteine is essentially wasted, as it is lost from the cycle. Previous experiments show that mice have significant levels of CBS protein and activity even when given a diet entirely devoid of methionine.³⁹ In contrast, in *Tg-I278T Cbs^{-/-}* mice, the lack of transsulfuration prevents the loss of methionine and, thus, they are able to better utilize the growth-limiting quantities in the MRD. Given this result, one might speculate that if a mammal was faced with a dietary environment in which methionine was specifically rate-limiting for growth (i.e., cysteine was available from other sources), mutations in the CBS allele might be commonplace.

Betaine-supplemented diet

The final diet we examined was a BSD.⁴⁰ In these experiments, betaine was dissolved in drinking water at a concentration of 20 g/L. Since the average mouse drinks about 5 mL of water per day, this amount of betaine corresponds to a dosage of approximately 4000 mg/kg, which would be equivalent to about 324 mg/kg/day when normalized by body-surface area.⁴¹ This concentration falls well within the 160–423 mg/kg/day range of betaine that is used to treat human CBS-deficient patients.^{13,42}

The mean tHcy in the BSD *Tg-I278T Cbs*^{-/-} mice was 209 μ M, but this was still significantly lower than the 349 μ M observed in RD mice (Fig. 4). Similarly, serum methionine levels in *Tg-I278T Cbs*^{-/-} mice were elevated compared to RD animals (373 vs. 157 μ M, $P < 0.0001$). In control mice, we observed a small but statistically significant reduction of tHcy levels (8.7 vs. 5.3 μ M, $P < 0.001$), but no difference in methionine (83 vs. 86 μ M, $P = \text{ns}$). In these studies, tCys was not measured.

With regard to other phenotypes, the effect of BSD was mixed. It was somewhat effective in reversing the facial alopecia phenotype (Fig. 6), but not as effective as MRD. BSD elevated liver SCD-1 protein levels, but only partially reversed the weight loss phenotype in male mice (Fig. 5A). DEXA confirmed a slight increase in both the fat and lean mass, but the difference was only statistically significant for the fat mass (Fig. 5B). BSD had no beneficial effects with regard to bone mineral density (Fig. 5C). Also, it should be noted that, with the exception of the alopecia phenotype, none of the beneficial effects of BSD were observed in female mice.

In control mice, BSD resulted in a significant inhibition of weight gain, although the magnitude was somewhat less than that observed in MRD (Fig. 5A). DEXA analysis indicated that this reduction of mass was due to a loss of both fat and lean mass (Fig. 5B; see Ref. 37). Bone mineral density was also reduced in the *Tg-I278T Cbs*^{+/-} BSD group compared to the RD group (Fig. 5C).

Gene–diet interaction effects

In each of the dietary interventions described above, the effects of the intervention seemed to vary significantly depending on the animals' genotype. The effects of genotype, diet, and their interaction on a single variable can be quantified using two-factor analysis of variance (ANOVA). We have performed such an analysis on two variables: fat mass and bone mineral density. In both cases, about 50% of the total variance in each variable could be explained by three factors: diet, genotype, and their interaction. All three factors were highly significant. For fat mass, diet accounted for 15.9%, genotype 7.6%, and the interaction for 24% of the variance. For bone mineral density, the breakdown was 28.5% diet, 9.8% genotype, and 13.9% interaction. The finding that the interaction effect is of the same order as each of the single effects indicates the strength of the gene–diet interaction.

Another way to consider this is to examine the response to diet when all the animals are considered without regard to genotype. For example, when we examine the relationship between RD, MRD, and fat mass in the entire cohort of mice, one would conclude that MRD increases fat mass (see Fig. 7). However, when one breaks down the cohort by genotype, a

dramatically different picture emerges. In *Cbs*^{+/-} animals, MRD decreases fat mass, while in *Cbs*^{-/-} mice, it increases fat mass. Thus, in this case, the gene–diet interaction is sufficiently strong as to change not only the magnitude but also the direction of the diet effect.

Conclusions

In this review, we have discussed the *Tg-I278T Cbs*^{-/-} mouse model of CBS deficiency and how it responds to long-term treatment with four different diets that vary in sulfur amino acids. On the basis of our data, we would rank the diets as follows (from best to worst): MRD > BSD > RD, NAC. In terms of human treatment, our data suggests that restricting methionine intake is probably the most effective way to keep tHcy levels low, but that betaine might also be useful, especially in patients who have trouble complying with a low-methionine diet. However, our data also suggests that cysteine supplementation may not be necessary in patients.

The other interesting finding was that control *Tg-I278T Cbs*^{+/-} mice responded entirely differently with regard to diet. In these, mice we would rank the diets differently: RD > BSD > MRD, NAC. These findings show how powerful gene–diet interactions can be and suggest that more thought should be given to potential interaction effects when interpreting the results of nutritional intervention or genome-wide association studies.

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Figure 1. Methionine cycle and transsulfuration pathway. THF, tetrahydrofolate; CH₂-THF, methylene-tetrahydrofolate; CH₃-THF, methyl-tetrahydrofolate; MS, methionine synthase; BHMT, betaine-dependent homocysteine methyltransferase; CBS, cystathionine β synthase; CGL, cystathionine γ lyase

Figure 2. *Tg-I278T Cbs^{-/-}* phenotypes. Original data taken from Ref. 21. In all panels, only males are shown, although females show similar behavior.²¹ (A) Photograph of *Tg-I278T Cbs^{-/-}* (asterisk) and sibling *Tg-I278T Cbs^{+/-}* mice, shown at 120 and 210 days. (B) Weight of *Tg-I278T Cbs^{-/-}* and sibling *Tg-I278T Cbs^{+/-}* mice. (C) Serum tHcy, methionine, and tCys levels. (D) Fat mass as determined by DEXA. (E) Bone mineral density as determined by DEXA. All error bars are 95% CI.

Figure 3.
Flow chart of experimental strategy.

Figure 4.

Diet, genotype, and serum metabolites. Data for this figure were compiled from previously published data.^{21,36,40} Only males are shown, but females behave similarly. (A) Bar graph of tHcy levels organized by diet and genotype. RD, regular diet; NAC, *N*-acetyl L-cysteine supplemented diet; MR, methionine-restricted diet; BSD, betaine-supplemented diet. Error bars show 95% CI. (B) Same as above, but serum methionine is shown instead. (C) Same as above, but serum tCys is shown.

Figure 5.

Weight gain, fat mass, and bone mineral density. (A) Growth curves of animals on indicated diets (blue, RD; black, NAC; red, MRD; green, BSD). Genotype is indicated at the bottom of the graph. (B) Total fat mass as determined by DEXA. Same color scheme as above. (C) Bone mineral density as determined by DEXA. Error bars in all graphs show 95% CI.

Figure 6.

Alopecia and diet. Each picture shows a sibling pair of 150-day-old mice on the indicated diet. The asterisk indicates the *Tg-I278T Cbs*^{-/-} animal. The other animal is *Tg-I278T Cbs*^{+/-}. Data for this figure compiled from previously published data.^{21,36,40}

Figure 7. Gene-diet interaction and fat mass. Graph shows fat mass in RD and MRD either unstratified or stratified by genotype. Error bars show 95% CI. Data for this figure compiled from previously published data.^{21,36}