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SUPPLEMENTARY INFORMATION

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Mutations in *SELENBP1*, encoding a novel human methanethiol oxidase, cause extraoral halitosis

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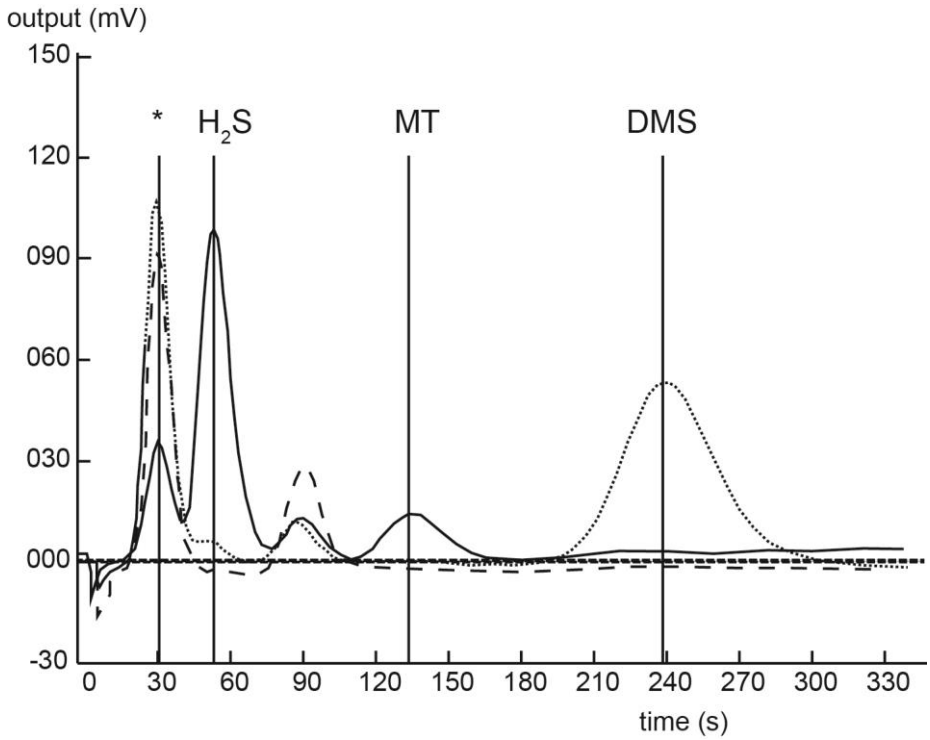


Supplementary Figure 1

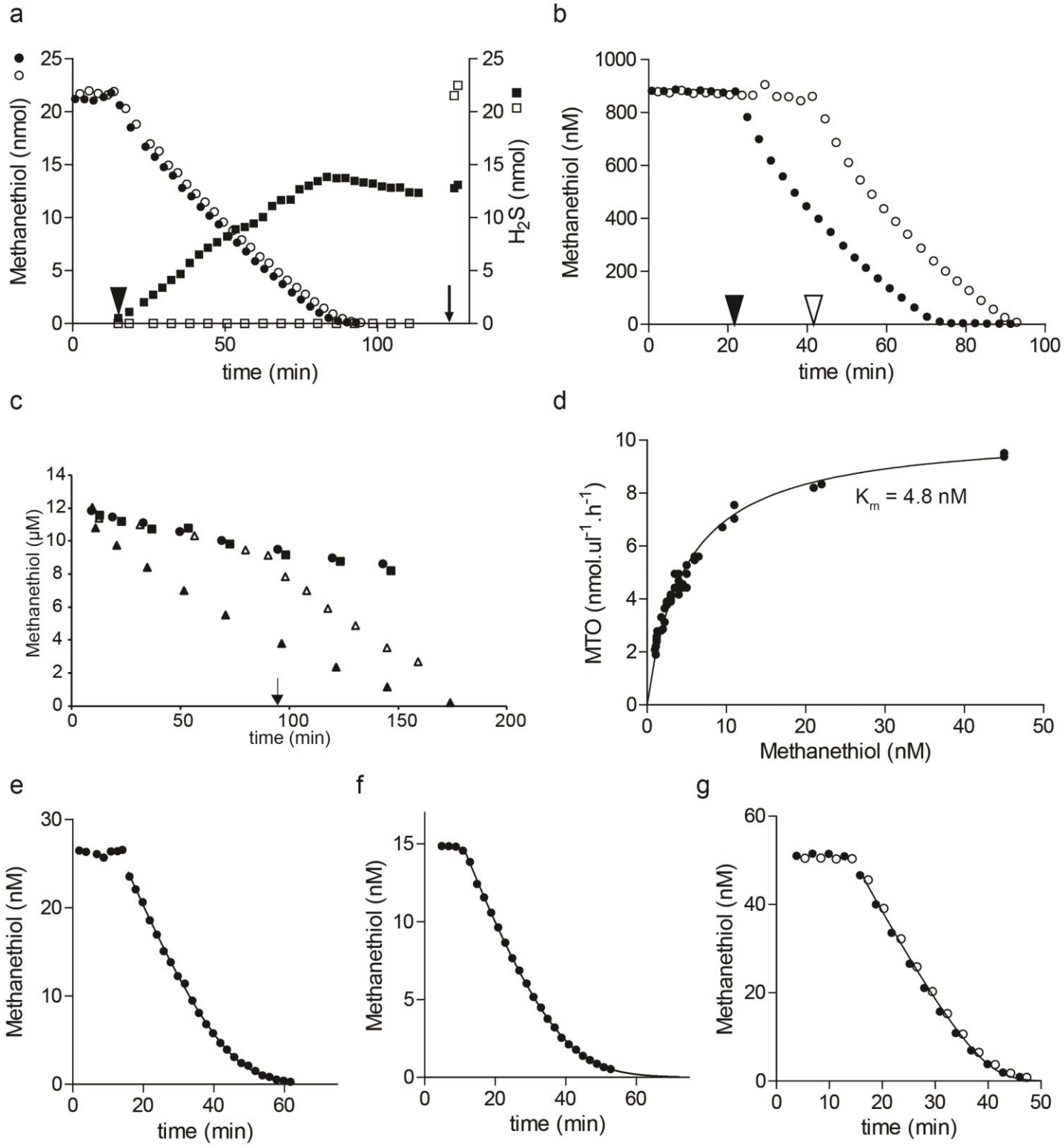
Breath analysis in the dental clinic.

Breath samples of the extra-oral halitosis patient CII-2 (dotted line), a control person (dashed line), and an intra-oral halitosis patient (solid line) analysed in a portable gas chromatograph (OralChroma^{TM}). Vertical lines indicate the retention times of H_2S, MT and DMS. The vertical line indicated with * is a non-sulfur volatile background peak as described in: Hanada, M. *et al.* Portable oral malodor analyzer using highly sensitive In2O3 gas sensor combined with a simple gas chromatography system. *Analytica Chimica Acta* **475**, 27-35 (2003).

Supplementary Figure 2

Alignment of the *Hyphomicrobium* strain VS MT-oxidase and the human SELENBP1.

The full-length alignment was performed using the Pairwise Alignment tool of the Protein Information Resource (http://pir.georgetown.edu/pirwww/search/). The Smith-Waterman score was 398. The proteins show 26.0% identity (highlighted in yellow and by *) and 54.2% similarity (highlighted in green) in 461 aa overlap. The signal peptide of the bacterial enzymes is underlined. In red putative TTQ residues (W) and copper ligands (H).



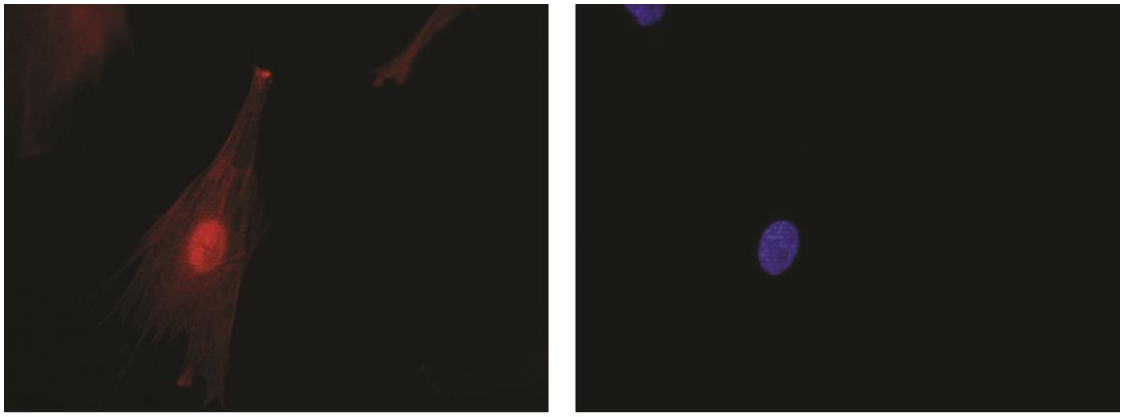
Supplementary Figure 3

Newly established assay for MTO activity.

a) MT oxidation results in H_2S production. The amounts of MT (circles) and H_2S (squares) were followed in time. Two standard assay mixtures (in 250 ml serum bottles) containing MT with- (closed symbols) or without (open symbols) Zn (0.2 mM) were started by the

addition of 2 µl human control erythrocyte extract as a MTO source at the time point indicated by the arrowhead. Without Zn in the medium the molar amount of MT that has been converted is seemingly higher that the amount of H_2S formed. Zn-ions will capture formed H_2S. After MT was depleted the reaction mixtures were acidified thus releasing the H2S again (indicated with an arrow). In the incubation mixture with Zn the amount of sulfide formed is equimolar to the amount of MT that has been converted. In the absence of Zn-ions the formed H_2S can be further enzymatically metabolized towards thiosulfate (Ref) thus explaining the lower molar recovery of H_2S in the absence of Zn-ions. b) Kinetic analysis of the MTO assay. Two identical standard assays using 10 µl human control erythrocyte extract were started at t = 0 under anaerobic conditions. The closed arrowhead indicates the addition of 20 ml of oxygen to incubation indicated with ●. The open arrowhead indicates the addition of oxygen to the other incubation (○) twenty minutes later. c) MTO activity in colon cancer cell lines HT29 (▲) and SW480 (■). Oxygen dependence of the reaction in 3 ml exetainers is shown with cell line HT29 under anoxic condition (△), arrow indicates the addition of 0.25 ml of oxygen. Background disappearance of MT (●). Each data point is the average of duplicate incubations that showed less than 5% difference. d) Michaelis-Menten plot of MTO activity measurements done with control erythrocyte extract using different substrate concentrations. The apparent K_m is indicated. e-g) Kinetic analysis of MTO activity in human erythrocyte extract (e), HT29 colon cancer cell extracts (f) and wild type mouse liver extracts (g, duplicate measurements as open and closed symbols) under low starting concentrations of MT. In each panel the line is fitted on the data using Michaelis-Menten kinetics. This resulted in apparent Km values of 5 nM, 4.7 nM and 6 nM, respectively.

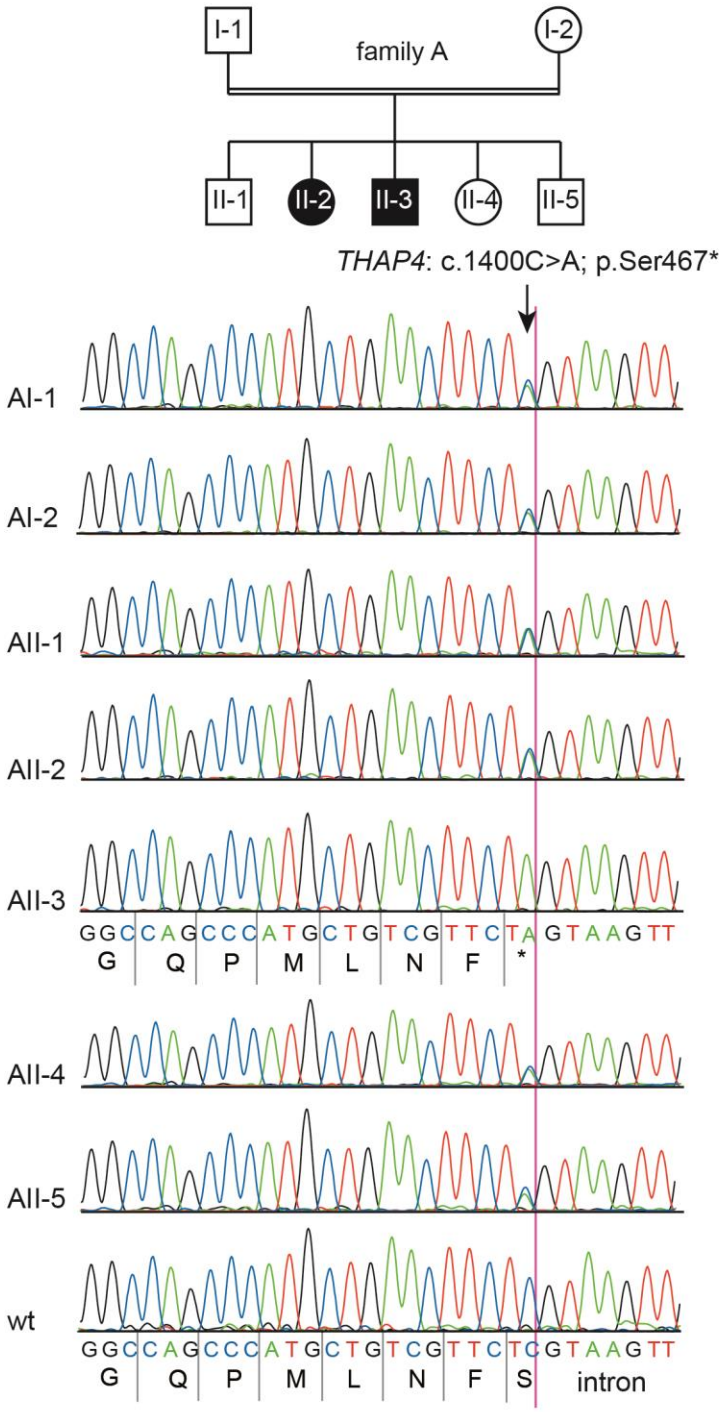
Ref: Szabo, C. *et al.* Regulation of mitochondrial bioenergetic function by hydrogen sulfide. Part I. Biochemical and physiological mechanisms. *Br J Pharmacol* **171**, 2099-122 (2014).



Supplementary Figure 4

Intracellular localization of SELENBP1.

Stably transduced patient cells were stained for indirect fluorescence analysis with anti-V5 antibodies that detect the C-terminal tag of SELENBP1 (red). Nuclei were stained with Hoechst dye (blue).



Supplementary Figure 5

THAP4-mutation analysis in pedigree A.

Sanger sequencing of the *THAP4* mutation in family A shows that the c.1400C>A mutation occurs in homozygous form in the male individual AII-3 with the malodour and the neurological features while in heterozygous form in the female individual AII-2 with the malodour syndrome without neurological features. None of the other 3 sibs in this family has this *THAP4* loss of function mutation in homozygous form. Black symbols in the pedigree indicate the malodour in individuals AII-2 and AII-3.

SUPPLEMENTARY NOTE

DETAILED DESCRIPTION OF THE FAMILIES

Family A

Patient AII-3 is the third child of healthy consanguineous parents of Turkish origin living in Germany. Since his birth a cauliflower-/cabbage-like bad breath has been noted which has resulted in major contact problems from kindergarten on and was reported to be more pronounced when fever was present. School enrollment was postponed because of inadequate total development including a mild cerebral movement disorder. After a journey in Morocco the patient developed pneumonia of the left inferior lobe associated with a pleural effusion and hemothorax. Parts of the lung were removed. The patient was further examined at the age of 15.5 years following recurrent febrile periods with muscle and joint pain and subsequent abdominal cramps and weight loss of 14 kg. Hepatosplenomegaly was noted. Because of his Marfanoid appearance an ophthalmologic examination and further cardiac investigations were performed but yielded no diagnostic abnormality. Genetic investigations provided no evidence for familial Mediterranean fever and deficiency of glucose-6-phosphate dehydrogenase was excluded. IQ testing revealed a value in the lower normal level but the patient attended a regular school. At age 28 years he works as a machine operator and he has married. Over the years many metabolic investigations have been performed to identify a possible inborn error of metabolism. Metabolite data provided no evidence for methionine adenosyltransferase deficiency or tyrosinemia. Cerebral MR spectroscopy has shown no structural abnormalities except for enlarged perivascular spaces (Robin Virchow). MR spectroscopy of the brain detected no abnormalities. While amino acid analysis yielded no diagnostic abnormality, analysis of urinary organic acids consistently resulted in an unusual signal of dimethyl sulfone. Remarkably, dimethyl sulfone excretion was also observed in the oldest sister (patient AII-2). It was not present in the urine samples of the parents, the other siblings and other family members. Patient AII-2 has the same malodor as her brother. In contrast to her brother she has an uneventful medical history. She is the mother of two healthy children without malodor. *SELENBP1* mutations were found in both sibs thus explaining their malodor. In an attempt to explain the neurological phenotype in the male sibling whole exome analysis was performed. No mutations were found in genes that are known to cause neurological signs and symptoms. Homozygous loss of function mutations caused by a premature stop codon in the genes *OR4S2* and *THAP4* were identified. No diseases have been connected to these genes. The mutation of the gene encoding an olfactory receptor (*OR4S2*) is very unlikely to be responsible for the additional neurological phenotype. However, we further followed up on the homozygous non-sense mutation in *THAP4* (c.1400C>A, p.S467*) encoding a member of a new family of DNA-binding proteins. Segregation analysis demonstrated that none of the siblings of patient AII-3 carried the mutation in the homozygous state (Supplementary Figure 5). *THAP1* mutations are known to cause a neurological

phenotype (torsion dystonia-6; MIM: 602629). We consider *THAP4* a possible candidate gene responsible for the broader neurological phenotype in the male patient. The sister of this patient has the malodour but no neurological features. She has bi-allelic mutations *SELENBP1* but not in *THAP4*.

Family B

Patient BII-2 was born to consanguineous Portuguese parents. He came to our attention at the age of twelve, due to a cabbage-like breath odor. His father died at the age of 38 years after 7-months of progressive bulbar and limb weakness, diagnosed as amyotrophic lateral sclerosis (ALS). Patient BII-2 had mild developmental delay (walked at 18 months, first words at 2 years) and learning difficulties. His abnormal breath was first noticed at the end of his first year of life and was variable over the day and between different days. He used no medication and had a normal Portuguese diet and there seemed to be no relationship between the malodor and the food content. The breath malodor was particular noticed when he was in a closed space (a car with closed windows for example). Urine and sweat had no particular smell. Besides his learning difficulties he also had mild dysmorphia including pectus carinatum and scoliosis. He had normal routine investigations including normal red blood count and normal liver function tests. Urine organic acids and plasma amino acids were reported normal including normal methionine levels in plasma. At 15 years of age the patient developed rapidly progressive weakness of the legs evolving to generalized weakness, muscular atrophy with twitching and cramping of muscles. EMG revealed normal nerve conduction velocity. Brain and spinal cord MRI were unremarkable. Screening for mutations in the *SOD1* gene by fluorescent DNA sequencing of all 5 exons and adjacent splice sites was negative. Severely progressive ALS was diagnosed and he died at 16.5 years.

Family C

Patient CII-2 was born to healthy non-consanguineous Dutch parents. She has normal intelligence and her halitosis first became obvious to her in her student period. The cabbage odor-like smell was observed by her friends worsening after drinking beer. She was referred to the Clinic for Periodontology in Amsterdam for diagnosis and therapy. She had a dry mouth, healthy periodontium, no tongue coating and a bad taste in her mouth. During the halitosis examination the air from her nose and mouth had an unpleasantly sweet smell resembling dimethyl sulfide (DMS). Based on the organoleptic score and results of gas chromatography measurements (OralChroma^{TM}) she was diagnosed as having extra-oral halitosis due to an increased dimethyl sulfide concentration in her breath (Supplementary Figure 1). DMS is a byproduct of mashing and fermentation, so it is present to some degree in all beers. Her sweat did not smell specifically. The patient is in overall good health at the age of 36 years. Apart from the halitosis the patient has bilateral ptosis. Family investigations revealed that the healthy brother of the index patient also has increased DMS in plasma and in breath.

SUPPLEMENTARY RESULTS AND DISCUSSION

Malodor diagnosis in specialized dental clinics

The bad breath of the patients in this study is the most pronounced and unifying clinical characteristic of this malodor syndrome. They have an extra-oral halitosis as the formation of the sulfur-containing metabolites occurs in their endogenous metabolism. These metabolites arrive in the breath via the blood and the lungs. Specialized dental clinics use portable gas chromatography to detect the origin of oral malodor (OralChroma^{TM}). Using this technique increased DMS breath levels could be demonstrated in patient CII-2 (Supplementary Figure 1). Due to limitations in sensitivity of this technique the increased MT concentration in the breath of patient CII-2 could not be detected. The figure shows that increased MT levels could be demonstrated in patients with oral halitosis, in whom the MT is formed by bacteria located in the mouth cavity. The genetic defect described in this study adds to the differential diagnosis of malodor in these specialized dental clinics.

Frequency analysis of SELENBP1 mutations

The frequency at which SELENBP1-mutations may cause extra-oral halitosis is unknown. However with the availability of the Exome Aggregation Consortium (ExAC) Data Set, containing exome data of 60,706 unrelated individuals sequenced as part of various disease-specific and population genetic studies, these frequencies can be estimated. We extracted all "PASS" filter variants from individuals in the coding regions and canonical splice sites (+/-2bp adjacent to the exon boundaries) for the genes *CFTR*, *PAH* and *SELENBP1* from ExAC v0.2. This resulted in 1003, 290 and 309 variants for *CFTR*, *PAH* and *SELENBP1* respectively. Similarly we collected all variants for these genes that were identified in our in-house cohort of about 12,243 exomes of healthy parents and unrelated patients with a variety of different disorders. This resulted in 312, 110 and 116 variants in the coding regions for *CFTR*, *PAH* and *SELENBP1* respectively (all variants listed in Supplementary Table 1). In order to estimate the bi-allelic frequencies, we first compared the coverage of coding regions and canonical splice sites for the three genes in ExAC. Although ExAC adjusts the allele frequencies based on the coverage of the corresponding positions, this is only possible if positions are not systematically poorly covered. We considered a position to have insufficient coverage if the coverage was less than 10x in more than 90% of the samples. Whereas *SELENBP1* and *CFTR* were fully covered according to this criterion, *PAH* was covered for only 96.77%. We used this number to correct our estimates of bi-allelic frequencies for *PAH* by dividing the total frequency of selected variants by 0.9677.
Knowing the frequencies for CF and PKU we calculated the optimal CADD PHRED score for which selection of variants with this score or higher, would result in bi-allelic frequencies for CFTR and PAH that corresponded best with the known frequencies (Cystic fibrosis 1:3,650; PKU 1:18,000) [66,67]. In addition we excluded variants that regardless of the CADD score were very frequent, based the number of alleles in the cohort. We optimized the maximum number of alleles together with the

CADD PHRED score such that bi-allelic frequencies for CFTR and PAH corresponded best with the known frequencies. The optimal cutoffs in the ExAC cohort were a minimum CADD score of 18 and maximum allele count of 90 (variants with higher frequencies were considered polymorphic), giving rise to an estimated frequency of 1 in 3,287 for CF and 1 in 20,064 for PKU. Correcting for the average of the deviation of these values from the actual frequency resulted in an estimate of 1 in 89,306 for bi-allelic mutations in *SELENBP1*.

We repeated this analysis based on the data from our in-house exomes, assuming the same coverage as in ExAC. This resulted in a CADD score of 20 and allele count of 40 which gave rise to a bi-allelic frequency of 1 in 3,902 for *CFTR* and 1 in 18,126 for *PAH*. After correcting for the average deviation of our estimates we found an estimated frequency of 1 in 79,948 for bi-allelic mutations in *SELENBP1*. This estimate is very much in range with the estimate from ExAC, and likely slightly lower because of the less stringent quality filtering of variants from our in-house cohort. This is also reflected in the higher optimal CADD score that corresponded to the known *CFTR* and *PAH* bi-allelic frequencies. We conclude that MTO deficiency due to mutations in *SELENBP1* will occur frequently. MTO deficiency may be a frequent inborn error of metabolism. If heterozygous individuals would also have increased concentration of DMS and other biomarkers of MTO deficiency as in the *SELENBP1* KO-mouse model mutations in *SELENBP1* may be responsible for a substantial percentage of extra-oral halitosis. Further studies on human carriers of the *SELENBP1* defect are required to clarify this.

The MTO activity assay

In the MTO activity assay the decrease in MT concentration in the headspace of closed bottles is measured over time (Supplementary Figure 3A). On a molar basis the H_2S formed during the incubation amounted to 65% of the MT converted by MTO when using erythrocyte extract (Supplementary Figure 3A). When H_2S was trapped by adding 0.2 mM Zn ions in the incubation mixture 90-110% sulfide recovery could be demonstrated upon release of sulfide by acidification. The likely explanation is that H_2S normally is further enzymatically converted. The Zn ions trap the H_2S thus preventing further enzymatic conversions. The data convincingly show equimolarity between the substrate (MT) conversion and product (H_2S) formation. In analogy to the bacterial enzyme, MTO activity was strictly dependent on oxygen. In the presence of MTO but without the addition of MT no H_2S production was found (not shown). When incubation bottles were extensively flushed with nitrogen gas no MT consumption or H_2S production (not shown) was observed above background levels and addition of oxygen caused the reaction to start immediately (Supplementary Figures 3B and 3C).

Supplementary Table 2

Primer sequences used for *SELENBP1* expression profiling

forward	5'- GAACTGGCCTTTCTCCACAC-3'
reverse	5'- CATCCAGCAGCACAAAACC-3'

REFERENCES FOR SUPPLEMENTARY NOTE

66. Bobadilla, J.L., Macek, M., Jr., Fine, J.P. & Farrell, P.M. Cystic fibrosis: a worldwide analysis of CFTR mutations--correlation with incidence data and application to screening. *Hum Mutat* **19**, 575-606 (2002).
67. Verkerk, P.H. [20-year national screening for phenylketonuria in The Netherlands. National Guidance Commission PKU]. *Ned Tijdschr Geneeskd* **139**, 2302-5 (1995).