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# *ALOX5* variants associated with susceptibility to human pulmonary tuberculosis

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**The 5-lipoxygenase (*ALOX5*)-derived lipid mediators leukotrienes and lipoxins have regulatory functions in inflammation by modulating activities of immune cells and cytokine production. Recently, it was shown in *ALOX5*$^{-/-}$ mice that host control of *Mycobacterium tuberculosis* is regulated by 5-lipoxygenase (5-LO). *ALOX5* polymorphisms were genotyped in 1916 sputum-positive patients with pulmonary tuberculosis (TB) from Ghana and in 2269 exposed, apparently healthy controls. Polymorphisms of a variable number of tandem repeats (VNTR) of the *ALOX5* promoter and of the exonic non-synonymous variant g.760G>A were analysed by fragment length determination and fluorescence resonance energy transfer, respectively, and DNA sequencing. Mycobacterial lineages of >1400 isolates were differentiated biochemically and genetically. Carriers of one variant (*n* repeats $\neq$ 5) and one wild-type VNTR allele (*n* = 5) or of the exonic allele g.760A had a higher risk of TB [$P_{corrected}$ = 0.026, odds ratio (OR) 1.19 (95% CI 1.04–1.37) and $P_{corrected}$ = 0.026, OR 1.21 (95% CI 1.04–1.41), respectively]. The association of the exonic variant was stronger in infections caused by the mycobacterial lineage *M. africanum* West-African 2 [$P_{corrected}$ = 0.024, OR 1.70; (95% CI 1.2–2.6)]. Determination of haplotypes revealed the strongest associaton with TB for the 'non-5/760A' haplotype compared with the 'non-5/760G' haplotype (*P* = 0.003, OR 1.50). Our observation of an association of *ALOX5* variants with susceptibility to TB contributes evidence of the importance of 5-LO products to the regulation of immune responses to *M. tuberculosis*.**

## INTRODUCTION

Leukotrienes (LTs) and lipoxins (LXs) are of great significance in the generation of appropriate responses to inflammatory disease (1). The key enzyme in the biosynthesis of LTs and LXs from arachidonic acid is the 5-lipoxygenase (5-LO), encoded by *ALOX5*. LTs and LXs are involved in the regulation of immune cells and cytokine release. Whereas distinct classes of LTs ($LTC_4$, $LTD_4$, $LTE_4$; cysteinyl LTs) primarily affect the contractibility of airway smooth muscles and small vessels, $LTB_4$ and the class of LXs ($LXA_4$, $LXB_4$, ATL; aspirin-triggered LXs) regulate cells of innate and adaptive immune responses. $LTB_4$ is a pro-inflammatory mediator and attracts and stimulates polymorphonuclear leukocytes (PMN), monocytes, macrophages, natural killer (NK) cells and dendritic cells. Phagocytosis of microorganisms by alveolar macrophages and PMN was shown to be dependent on $LTB_4$ (2,3). A T-helper cell type

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1 immune response is supported by enhanced production of interferon (IFN)-γ and interleukin (IL)-12 (1,4).

The anti-inflammatory properties of LXs antagonize those of LTs in innate immunity by inhibiting PMN and NK cell functions, suppressing IL-12 release (4) and modulating the immune response by stimulation of IL-4 production (5), while blocking IL-5 and IL-13 and inhibiting eosinophil effector functions (6). In support of their anti-inflammatory mission, LXs exert also pro-resolving effects in inflammation and aid to clear microbial invaders by stimulating nonphlogistic phagocytosis of apoptotic PMN by macrophages (reviewed in 7). A strong relation of LXs and $LTB_4$ with the control of cytokine/chemokine networks and immune cells exists, suggesting efficient regulation of either the propagation of inflammatory disease by $LTB_4$ or resolution of inflammation by LXs.

Susceptibility to tuberculosis (TB) is considered to depend on human polygenic variability. However, only a few genetic variants have so far been reported to be associated with susceptibility or resistance to TB, and these variants exert moderate effects only (8). Recently, it was shown in 5-LO deficient (*ALOX5*$^{-/-}$) B6129SF2/J mice that host control of *Mycobacterium tuberculosis* is strongly influenced by 5-LO (9). After infection with the *M. tuberculosis* H37Rv strain, low background levels of $LXA_4$ and $LTB_4$ were measured in *ALOX5*$^{-/-}$-mice compared with high levels in *ALOX5* wild-type (*ALOX5*$^{WT}$) mice, with $LXA_4$ persisting for a considerably longer period than $LTB_4$. Mycobacterial loads were significantly lower in *ALOX5*$^{-/-}$ than in *ALOX5*$^{WT}$ mice, resulting in the containment of inflammation and extended survival. Furthermore, significant differences in the expression of IL-12, IFN-γ and iNOS were found with higher levels in *ALOX5*$^{-/-}$ mice. Treatment with the synthetic aspirin-triggered LX analogue ATLa2 resulted in complete reversal of the protection that was conferred by 5-LO deficiency (9).

*ALOX5* comprises 14 exons and 13 introns approximately 82 kb on chromosome 10 (10q11.2; http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=152390; accessed November 7, 2007). The promoter is GC-rich, and the region between −179 and −56 bp is essential for gene expression (10). In that region, a variable number of tandem repeats (VNTR) has been identified, consisting of $[5'\text{-GGGCGG-}3']_{2-8}$, which are targets for binding of the transcription factor Sp1. Insertions or deletions of the Sp1-binding motif have created variant alleles of two to eight repeats. Depending on the number of repeats, Sp1 binding and subsequent gene expression are modified (11,12). In all populations and ethnicities studied so far, alleles of five 5′-GGGCGG-3′ Sp1-binding motifs at positions −176 to −147 bp occur at highest frequencies and are considered the common wild-type. The frequencies of variant VNTR genotypes differ significantly between populations. In Caucasians and Asians, whereas the common '5/5' VNTR genotype occurs at frequencies of 55–77 and 30–38%, respectively, the '3/5' genotype is with 40% the most frequent genotype among Afro-Americans (12–16). The number of repeats was shown to affect the efficacy of pharmacological 5-LO inhibition and cysLT-receptor antagonizing drug-treatment response in asthma (14,15) and the severity phenotype of asthma (12,13). In atherosclerosis, VNTR polymorphisms were associated with increased intima-media thickness (16).

On the basis of the mouse experiment (9) we hypothesized that naturally occurring *ALOX5* variants might also be relevant to the phenotype of human *M. tuberculosis* infection. We have in an association study genotyped the promoter VNTR polymorphism and a non-synonymous exonic variant. Any influence exerted by these variants on susceptibility to TB should be reflected in a large sample of active sputum-positive cases with pulmonary TB that we recruited in Ghana, West Africa, and compared it with a control group of substantial size.

## RESULTS

### Power of the association study: Hardy–Weinberg equilibrium

A power of detection >90% was achieved for both multiplicative and additive models, assuming an approximative TB prevalence of 0.004 in West Africa, a frequency of 0.1 for high risk alleles and a genotype relative risk of 1.3 ($\alpha = 0.05$) with our sample size (case–control ratio = 1.18). The distribution of the exonic polymorphism that was tested and that of the promoter VNTR polymorphism were in Hardy–Weinberg equilibrium in cases and controls.

### *ALOX5* promoter VNTR polymorphism

The VNTR polymorphism in the *ALOX5* promoter was genotyped in 1820 cases and 2063 controls. The distributions of the variant alleles and genotypes are given in Tables 1 and 2. Among the five alleles and 13 genotypes that were identified were four genotype combinations ('2/2', '2/3', '2/4', '3/6') that were previously unrecognized. All genotypes that we found were confirmed by re-typing and DNA sequencing in at least two independent samples for each genotype. No significant differences were found when frequencies of alleles between cases and controls were compared.

Based on the observation that, in reporter gene assays in HeLa cells, any number of Sp1-binding motifs other than five ('non-5') was associated with decreased *ALOX5* expression, we followed the suggestion to combine for further analyses the genotypes in groups of the homozygous wild-type genotype '5/5', the heterozygous variant genotype '5/non-5' and the homozygous variant genotype 'non-5/non-5' (11,15). In the combined analysis, the genotype group '5/non-5' was significantly more frequent among cases than among controls [$P_{c2} = 0.026$, odds ratio (OR) 1.19 (95% CI 1.04–1.37)] (Table 2).

### *ALOX5* exonic variant

Genotyping of the non-synonymous exonic variant g.760G/A (Glu254Lys) was performed in 1916 cases and 2269 controls. The distributions of the g.760A and g.760G alleles and of the corresponding genotypes are given in Tables 3 and 4. The allele g.760A was slightly, but significantly associated with a higher risk of TB [$P_{c2} = 0.026$, OR 1.21 (95% CI 1.04–1.41)]. Stratification by mycobacterial lineages (Fig. 1)

**Table 1.** Distribution of the VNTR alleles among cases and controls

| VNTR alleles | *n*, % | | | Cases versus controls | | |
|---|---|---|---|---|---|---|
| | Cases ($n = 1820$) | Controls ($n = 2063$) | Total ($n = 3883$) | OR | 95% CI | $P_{c2}$ |
| 2 | 2, <0.1 | 6, <0.2 | 8, <0.2 | 0.38 | 0.04–2.11 | ns |
| 3 | 1584, 43.5 | 1778, 43.1 | 3362,/ 43.3 | 1.02 | 0.93–1.11 | ns |
| 4 | 653, 17.9 | 809, 19.6 | 1462, 18.8 | 0.90 | 0.80–1.00 | ns |
| 5 | 1352, 37.1 | 1484, 36.0 | 2836, 36.5 | 1.05 | 0.96–1.15 | ns |
| 6 | 49, 1.3 | 49, 1.2 | 98, 1.3 | 1.14 | 0.76–1.69 | ns |

$P_{c2}$, *P*-value corrected for the two genetic variants tested; ns, not significant.

**Table 2.** Distribution of the VNTR genotypes among cases and controls

| VNTR genotypes/groups | *n*, % | | | Cases versus controls | | |
|---|---|---|---|---|---|---|
| | Cases ($n = 1820$) | Controls ($n = 2063$) | Total ($n = 3883$) | OR | 95% CI | $P_{c2}$ |
| non-5/non-5 | 762, 41.9 | 939 / 45.5 | 1701 /43.8 | 1 | | |
| 2/2[a] | 0 / 0 | 2, <0.1 | 2, <0.1 | | | |
| 2/3[a] | 1, <0.1 | 1, <0.1 | 2, <0.1 | | | |
| 2/4[a] | 1, <0.1 | 1, <0.1 | 2, <0.1 | | | |
| 3/3 | 419, 23.0 | 492, 23.8 | 911, 23.5 | | | |
| 3/4 | 159, 8.7 | 227, 11.0 | 386, 9.9 | | | |
| 3/6[a] | 14, <0.8 | 17, <0.9 | 31, <0.8 | | | |
| 4/4 | 165, 9.1 | 193, 9.4 | 358, 9.2 | | | |
| 4/6 | 3, <0.2 | 5, <0.3 | 8, <0.3 | | | |
| 6/6 | 0, 0 | 1, <0.1 | 1, <0.1 | | | |
| 5/non-5 | 764, 42.0 | 764, 37.0 | 1528, 39.4 | 1.19 | 1.04–1.37 | 0.026 |
| 3/5 | 572, 31.4 | 549, 26.6 | 1121, 28.9 | | | |
| 4/5 | 160, 8.8 | 190, 9.2 | 350, 9.0 | | | |
| 5/6 | 32, 1.8 | 25, 1.2 | 57, 1.5 | | | |
| 5/5 | 294, 16.1 | 360, 17.5 | 654, 16.8 | 0.99 | 0.82–1.19 | ns |

Promoter genotypes were grouped according to functional relevance (10,14). $P_{c2}$, *P*-value corrected for the two genetic variants tested; ns, not significant.
[a]Previously unrecognized VNTR genotype.

**Table 3.** Distribution of g.760G>A alleles among cases and controls

| Allele | *n*, % | | | Cases versus controls | | |
|---|---|---|---|---|---|---|
| | Cases ($n = 1916$) | Controls ($n = 2269$) | Total ($n = 4185$) | OR | 95% CI | $P_{c2}$ |
| g.760A | 368, 9.6 | 366, 8.1 | 734, 8.8 | 1.21 | 1.04–1.41 | 0.026 |
| g.760G | 3464, 90.4 | 4172, 91.9 | 7636, 91.2 | 1 | | |

CI, confidence interval; $P_{c2}$, *P*-value corrected for the two genetic variants tested.

**Table 4.** Distribution of g.760G>A genotypes among cases and controls

| Genotype | *n*, % | | | Cases versus controls | | |
|---|---|---|---|---|---|---|
| | Cases(n = 1916) | Controls ($n = 2269$) | Total ($n = 4185$) | OR | 95% CI | $P_{c2}$ |
| g.760AA | 22, 1.2 | 14, 0.6 | 36, 0.9 | 1.84 | 0.97–3.81 | ns |
| g.760AG | 324, 16.9 | 238, 14.9 | 662, 15.8 | 1.17 | 0.99–1.39 | ns |
| g.760GG | 1570, 81.9 | 1917, 84.5 | 3467, 83.3 | 1 | | |

$P_{c2}$, *P*-value corrected for the two variants tested; ns, not significant.

revealed that the allelic association was largely due to the occurrence of g.760A among TB cases associated with the lineage *M. africanum* West-African 2 [$P_{c12} = 0.024$, OR 1.70 (95% CI 1.20–2.60)].

According to the commonly accepted phylogeny of mycobacterial lineages, *M. africanum* West-African 2 and *M. bovis* are closely related, varying in a single marker [region of difference (RD) 702] only. Sequence differences

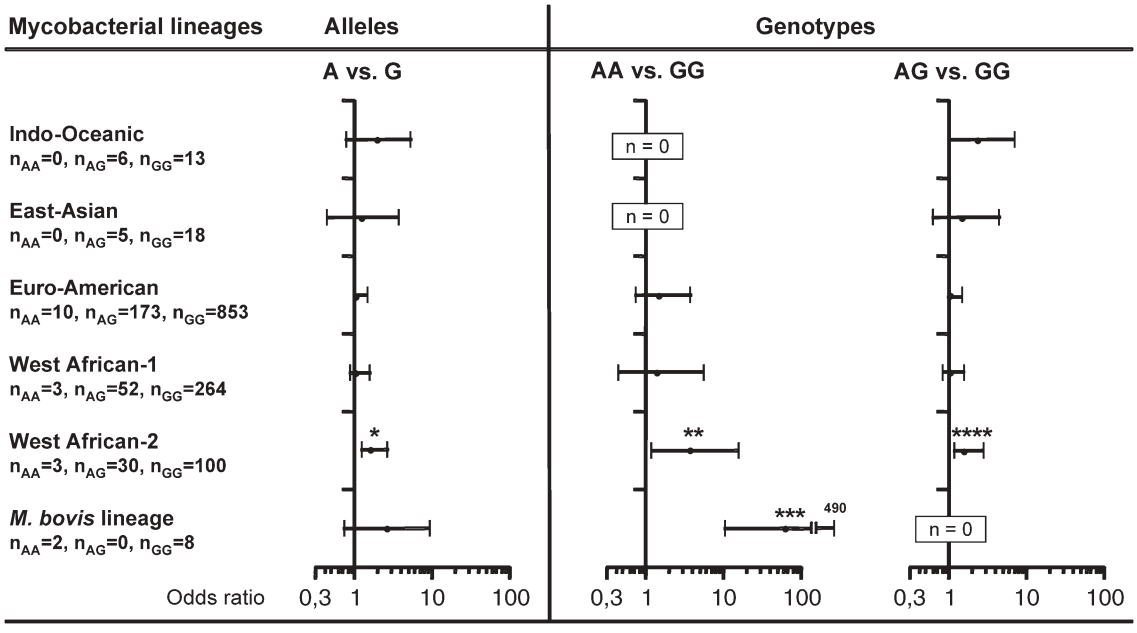


**Figure 1.** ORs and 95% confidence intervals (bars) for allele (g.760A) and genotype ('AA', 'AG') distributions among cases and controls ($n = 2269$) after stratification for mycobacterial lineages. *P*-values of genotypes were determined by logistic regression, adjusted for age, sex and ethnicity. $P_{c12}$, *P*-value corrected for two genetic variants tested and six mycobacterial lineages. $^{*}P = 0.002$, $P_{c12} = 0.024$; $^{**}P = 0.31$, $P_{c12}$, not significant; $^{***}P < 0.001$, $P_{c12} < 0.001$; $^{****}P = 0.012$, $P_{c12}$ not significant.

to other lineages are more complex (17). Based on the tight relationship of the two lineages and the low number of TB cases associated with *M. bovis* ($n = 10$), it appeared permissible to combine them in a group that is characterized by RDs 7, 8 and 10 (17). The variant g.760A was observed significantly more frequent among cases associated with either *M. africanum* West-African 2 or *M. bovis* than among controls [$P_{c12} = 0.006$, OR 1.85 (95% CI 1.27–2.65)].

The distribution of the g.760 genotypes 'G/G', 'A/G' and 'A/A' did not differ between cases and controls. When combining *M. africanum* West-African 2 and *M. bovis*, the 'A/A' genotype was strongly associated with a 7.3-fold higher risk of infection caused by either one of these two lineages [$P_{c12} = 0.0012$, OR 7.30 (95% CI 2.49–21.28); data not shown].

Notably, when assessing allele and genotype distributions seperately in males and females, the g.760A allele and the 'A/G' genotype were significantly more frequent in males with TB caused by the lineage West-African 2 compared with male controls [allele: $P_{c24} = 0.0096$, OR 2.08 (95% CI 1.33–3.17); genotype: $P_{c24} = 0.048$, OR 2.17 (95% CI 1.33–3.54); data not shown]. Associations with distinct mycobacterial lineages were not found in females.

### Haplotypes

When estimated haplotypes comprising the exonic alleles g.760G and g.760A and the grouped VNTR alleles 'non-5' and '5' were compared between cases and controls, the 'non-5/A' haplotype was significantly more frequent among cases than the 'non-5/G' ($P = 0.003$, OR 1.5) haplotype (Table 5). Stratification by mycobacterial lineages revealed a significant increase in the risk associated with the 'non-5/A' haplotype in *M. africanum* West-African 2 infections ($P_{c6} = 0.024$, OR 2.2).

## DISCUSSION

We found in a large Ghanaian study group of patients with pulmonary TB and healthy exposed controls that variant *ALOX5* alleles and genotypes were associated with susceptibility to TB. Our hypothesis of an influence of *ALOX5* variants in susceptibility to TB was derived from several *in vitro* and *in vivo* findings and from studies of mice infected with *M. tuberculosis*.

It has been shown in *in vitro* studies that pharmacological inhibition of the synthesis of 5-LO products abolished optimal killing of mycobacteria in human PMN infected with *M. bovis*, but was restorable by $LTB_4$ substitution (18). Impaired killing due to 5-LO inhibition applied also to *M. tuberculosis*-infected PMN (19). The significance of 5-LO is underlined by increased levels of $LTB_4$ in pleural exudates in human TB (20) and 7-fold higher levels of 5-LO and other factors involved in LT and LX synthesis in human alveolar macrophages, compared with peripheral blood monocytes (21).

Supporting evidence comes from animal experiments. In $ALOX5^{-/-}$ B6129SF2/J mice infected with *M. tuberculosis*, the pulmonary burden of mycobacteria was lower and the survival time substantially longer than in $ALOX5^{WT}$ mice (9). Inconsistent with this observation, however, were findings in infected BALB/c mice after pharmacological inhibition of 5-LO-dependent biosynthesis of LT and LX. 5-LO-inhibition caused suppression of efficient host control of *M. tuberculosis*, abundant bacterial loads, lower levels of IFN-γ and IL-12 and a shorter survival time compared with untreated mice (22).

**Table 5.** Distribution of estimated haplotypes among cases and controls

| Haplotype | Cases (%) | Controls (%) | Cases versus controls OR | *P*-value |
|---|---|---|---|---|
| non-5/G | 57.2 | 60.5 | 1 | 0.006 |
| non-5/A | 5.3 | 3.8 | 1.50 | 0.003 |
| 5/G | 33.0 | 31.5 | 1.11 | ns |
| 5/A | 4.5 | 4.2 | 1.14 | ns |

ns, not significant.

The findings in *ALOX5*$^{-/-}$ mice argue for protection against TB provided by low levels of anti-inflammatory $LXA_4$, and the findings in the pharmacological approach argue for protection of untreated mice provided by higher pro-inflammatory $LTB_4$ levels. Although the concentrations of $LXA_4$ and $LTB_4$ were reduced in 5-LO-deficient and 5-LO-inhibited mice, the impact of the beneficial reduction in $LXA_4$ synthesis versus the detrimentally decreased $LTB_4$ synthesis was different in the two studies. These seemingly contrasting consequences of 5-LO ablation might result from discrete disturbances of the 5-LO-dependent fine equilibrium of the stimulation of innate immune cells and Th1-promoting effects exerted by $LTB_4$ and antagonizing immunomodulation by $LXA_4$.

An estimation of interferences of the $LTB_4/LXA_4$ ratio is hypothetical. For LX synthesis, other enzymes (12-LO, 15-LO, COX-2) are required in addition to the essential 5-LO, leading to at least three pathways of LX generation. Kinetics, origins and effects of these enzymes may vary. In contrast, the generation of $LTB_4$ is solely restricted to the 5-LO pathway. A balanced homeostasis of $LXA_4$ and $LTB_4$ appears to be the critical determinant of relative susceptibility or protection. It is conceivable that the homeostasis is subjected to variation during different phases of the infection, namely during acute inflammation and subsequent resolution, and that infection, innate immune responses, pathology, adaptive immune responses and granuloma formation in TB are associated with differing levels of $LXA_4$ and $LTB_4$.

$LTB_4$ and $LXA_4$ levels appear to play a critical role in infections caused by *Toxoplasma gondii*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* as well (2,3,23,24). It is reasonable to assume that, for succeessful replication and survival, different pathogens require different $LTB_4$ and $LXA_4$ levels (24).

*ALOX5* gene expression depends on the copy number of the Sp1-binding sequences (VNTR) in the promoter, whereby the '5'-allele, compared with the '3', '4' and '6' alleles, was associated with the highest degree of expression (11). Our results are in line with the genotype distribution in an Afro-American study group (14), where the '3/5' genotype was also the most frequent one. In contrast, the '3'-allele was the predominant allele among our study participants.

After grouping the VNTR genotypes according to their influence on gene expression *in vitro* (11,14,15), the '5/non-5' genotype in our study population was associated with a discretely but significantly higher risk for TB compared with the 'non-5/non-5' genotype group. No difference was observed between the '5/5' and the 'non-5/non-5' genotype groups. The capacity of 5-LO in the cell to generate products may be modified according to the various genotypes, whereby reports are not available on *in vivo* enzyme activities of individual heterozygous genotypes. It is imaginable that, in a '5/non-5' genotype, the '5'-allele compensates with increased gene expression for the lower activity of 5-LO associated with a 'non-5' allele.

We found a high number of individuals with promoter genotypes of low numbers of Sp1-binding motif copies and an accordingly presumed low 5-LO activity, including the novel genotypes that were identified (Table 4). The association of these genotypes with a decreased TB risk argues in favour of selection for these variants, consistent with the observation that 5-LO deficiency in mice was associated with relative protection from TB. The speculative assumption of selection for genotypes of lower numbers of Sp1-binding motifs awaits to be confirmed. This need applies also to the lack of an association of the '5/5' genotype with susceptibility to TB by confirmation in epidemiological replication studies and measurement of 5-LO mRNA, $LTB_4$ and $LXA_4$ levels in individuals of different genotypes. The importance of further studies is evident, as there exist ambiguous *in vitro* results from reporter gene assays using Schneider cells, attributing the highest *ALOX5* expression to promoter VNTR alleles of six copies (25). Moreover, complete deletion of the tandemized Sp1-binding motifs caused a limited reduction of 41–47% in MM6 (Mono Mac 6) and HeLa cells, respectively (26). Nevertheless, most studies so far have applied the proposed grouping scheme of genotypes (11). Furthermore, experiments have been performed showing that *ALOX5*-mRNA expression was lower in individuals bearing two deletional variants (12).

Although the tandem repeat is absent in the mouse-derived monocyte-macrophage cell-line RAW 264.7 (27), future studies could also include mice experiments, as there exists in that cell-line an orthologous Sp1-binding site which is functionally important for gene expression. It is so far unknown whether the binding motif is in mice subjected to genetic variation and whether the absence of the VNTR polymorphism applies to all mice strains. If not, this would in part contribute to explain the opposite consequences of 5-LO inhibition in B6129SF2/J (9) and BALB/c mice (22). Taken together, the correlations of the number of Sp1-binding motifs with *ALOX5* expression are not fully understood yet.

In our study, the exonic allele g.760A (Glu254Lys) was associated with TB in the total case–control sample. Stratification for mycobacterial lineages revealed that the association was largely due to the high frequency of the allele g.760A in infections with the lineage *M. africanum* West-African 2. The association of the genotype 'A/A' with *M. bovis* infections appears to be of limited value due to the low number of these infections. However, when combining the closely related mycobacterial lineages of *M. africanum* West-African 2 and *M. bovis*, the ORs of both the allelic (g.760A) and the genotypic ('A/A') associations increased considerably ($1.7 > 1.85$ and $4.18 > 7.3$, respectively). It cannot be conclusively determined in how far the structural Glu254Lys substitution causes modifications of 5-LO activity and of the levels of its products. The altered charge and the steric

proximity of the variant to the phosphorylation site Ser271 of 5-LO (28) argue, though, for a possible influence. Whether there is any compensating advantage that might be provided by g.760A cannot be defined.

The geographical overlap of infections of the mycobacterial lineage *M. africanum* West-African 2 and the *ALOX5* allele g.760A is worth to note. In certain geographical areas, a limited number of dominant mycobacterial strains cause a disproportionately high number of TB cases. It has been shown that mycobacterial lineages that are prevailing in distinct populations strongly correlate with the ethnicity affected, and the host's region of origin has even been claimed to be predictive of the infective strain (29). These observations indicate co-evolution of hosts and pathogens (30) and are underlined by our finding of a close association between a mycobacterial lineage that is almost exclusive to Africa and an ethnicity-specific host genetic factor associated with disease. Based on the observation that distinct features of immunity to *M. tuberculosis* may depend on the infecting mycobacterial strain (31,32), distinct strains might also differentially activate human 5-LO.

It is established and re-verified by our consecutive enrolment of patients that the incidence of clinical TB is higher in males than in females. The reasons for the unequal risk are largely unclear. In addition to socio-economic conditions, host genetic factors have been suggested to be involved (33). We have found gender differences in the distribution of g.760A among cases associated with *M. africanum* West-African 2 and controls. Whether the different risk for male and female g.760A carriers after infection with *M. africanum* West-African 2 significantly contributes to the overall gender-specific risk is hypothetical. Although imaginable, gender-specific regulation of 5-LO activity has not been observed so far.

The 'non-5/G' haplotype, comprising the 'protective' alleles 'non-5' and g.760G occurred more frequently among controls, compared to all other haplotypes and with the 'non-5/A' haplotype being associated with a 1.5-fold higher risk for TB. The association was largely driven by the high prevalence of the exonic variant g.760A in infections caused by *M. africanum* West-African 2 as evident after stratification by mycobacterial lineages. In these infections, the haplotype 'non-5/A' was associated with a 2.2-fold risk of manifest clinical disease (data not shown).

At present, it cannot be defined whether the balance between $LXA_4$ and $LTB_4$ is affected by different *ALOX5* genotypes, in particular because additional pathways of LX biosynthesis exist that may be induced or downregulated when the obligatory 5-LO mechanism is affected. Notably, secretable enzymes with 15-LO activity have been identified in *P. aeruginosa* and *T. gondii* (34,35) that may influence LX production inside the host. Speculations about a 'lurking gene' in the *M. tuberculosis* genome that exhibits LO activity are intriguing (36).

The production of adequate amounts of LT and LX depends also on the dietary intake of arachidonic acid, the primary substrate of 5-LO and its metabolic precursors (37,38). Vice versa, the production of LTs and subsequent activation of monocytes and neutrophils are reduced when the dietary proportion of $n-3$ fatty acids is increased (39). Inuit who traditionally consume great quantities of marine $n-3$ fatty acids [eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA)] and lower amounts of $n-6$ fatty acids such as arachidonic acid are more prone to develop TB (40). A correlation of fatty acids, LTs and the risk of TB has also been demonstrated in guinea pigs (38). Newly identified classes of bioactive lipid mediators derived from EPA and DHA, the protectins and resolvins, may contribute to the risk of manifest TB and persistence of mycobacteria. Protectins and resolvins are partly dependent on 5-LO and exhibit, as do LXs, anti-inflammatory and pro-resolving properties (7,41).

A weakness of our study is the lack of information of individual *ALOX5* expression and 5-LO activities that can be compared between cases and controls. Moreover, our genetic findings await replication in independent study groups and more detailed analyses of the effects of *ALOX5* variants on the activity of 5-LO. If confirmed, the results would indicate that genetic variation of LT and LX dependent inflammatory responses may increase susceptibility to TB. Whether these findings will in the future be of value in the design of inflammation-modulating intervention strategies to be applied in the treatment of TB may not be foreseen.

## MATERIALS AND METHODS

### Ghanaian study group

Study subjects were recruited in Ghana, West Africa, between September 2001 and July 2004. The recruitment area and the enrolment procedure have previously been described (42). Briefly, patients were enrolled at the two major Teaching Hospitals in Accra and Kumasi and additional hospitals or policlinics in Accra, Kumasi, Obuasi, Agona, Mampong, Agogo, Konongo and Nkawie (Ashanti Region), Nkawkaw and Atibie (Eastern Region) and Assin Fosu and Dunkwa (Central Region). Characterization of patients included (i) documentation of the medical history on standardized structured questionnaires including self-reported duration of cough and symptoms of TB (dyspnoea, chest pain, night sweats, fever, haemoptysis, weight loss), (ii) two independent examinations of non-induced sputum specimens for acid-fast bacilli, (iii) determination of HIV serology and confirmation of positive results by an alternative test, (iv) culturing and subsequent differentiation of mycobacterial lineages and (v) a posterior–anterior chest radiography. Inclusion criteria were two sputum smears positive for acid-fast bacilli, no history of previous TB or anti-mycobacterial treatment and an age between 6 and 60 years. Exclusion criteria were incomplete information provided on the questionnaire, evidence of alcoholism, drug addiction and other apparent generalized disease. A total of 2004 patients fulfilled all criteria for participation.

Unrelated personal contacts of cases and community members from neighbouring houses of cases and public assemblies were recruited as controls. The principal criterium for enrolment was no history of TB or previous anti-mycobacterial treatment. Characterization of controls included a medical history, chest X-ray and a tuberculin skin test (Tuberculin Test PPD Mérieux, bioMérieux, Nürtingen, Germany). A total of 1231 personal contacts and 1135 community members fulfilled the criteria for participation

and were available as controls. In the total study group, the male to female ratio was 1:0.58 and the mean age of participants was 33 years without gender differences.

Study participants belonged to the following ethnic groups (cases/controls): Akan including Ashanti, Fante, Akuapem (63.8%/59.4%), Ga-Adangbe (14.8%/19.7%), Ewe (7.0%/9.3%) and ethnic groups of northern Ghana including Dagomba, Sissala, Gonja, and Kusasi (12.6%/10.4%). The proportions of ethnicities among patients and controls did not differ significantly.

The study protocol was approved by the Committee on Human Research, Publications and Ethics, School of Medical Sciences, Kwame Nkrumah University, Kumasi, and the Ethics Committee of the Ghana Health Services, Accra. Patients were treated according to the 'Directly Observed Treatment, Short-course' strategy organized by the National Tuberculosis Programme. Blood samples for genetic analyses and HIV testing were taken only after a detailed explanation of the study aims and written or thumb-printed consent for participation provided, including HIV testing. Disclosure of HIV test results was dependent on the documented willingness of participants to be informed and included referral to counselling and treatment provided by the Ghanaian AIDS Control Programme.

### Variants selected for genotyping

The selection of *ALOX5* variants for genotyping was based on (i) evidence for functional relevance and (ii) ethno-specific distribution patterns. First, the family of mutations consisting of insertions or deletions of the Sp1-binding 5′-GGGCGG-3′ motif (VNTR) in the *ALOX5* promoter was chosen for genotyping. Gene expression and Sp1-binding properties of this region differ depending on the number of repeats (11,12) and the VNTR is unevenly distributed between Caucasians and individuals of African origin. Second, the variant g.760G>A (Glu254Lys; http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=2228065), located in the sixth exon of *ALOX5*, was selected. The mutation results in a substitution of an acidic to a basic amino acid and structural modification of 5-LO. In addition, the distribution of variant alleles differs between Caucasians and individuals of African origin. In Caucasians, g.760G prevails with frequencies of almost 100%, whereas the g.760A allele occurs with frequencies of 3–8% in Afro-Americans and of 14% in Yoruba originating from Nigeria.

### Laboratory tests

The diagnosis of TB was made as described previously (42). *Mycobacterium* isolates were cultured on Löwenstein–Jensen media and shipped to the German National Reference Centre for Mycobacteria (Borstel, Germany) for analyses of biochemial, molecular and growth patterns. Molecular differentiation of mycobacterial lineages included spoligotyping and IS*6110* fingerprinting as described previously (43–45). Mycobacterial strains for further stratification were grouped according to the phylogenetic lineages described in Gagneux and Small (17).

For HIV-1/-2 testing, a capillary test system (Capillus, Trinity Biotech, Bray, Co Wicklow, Ireland) was applied. HIV positivity was confirmed by the Organon Teknika Vironostika HIV-1/-2 EIA (Organon Teknika, Turnhout, Belgium). The rate of confirmation was 100%.

### Genetic analyses

DNA was isolated from blood samples of study participants (AGOWA® mag Maxi DNA Isolation Kit, Macherey & Nagel, Germany) according to manufacturer's instructions.

Promoter variants were genotyped by multifluorescence fragment length determination in polyacrylamide gels. The fragment containing the Sp1 motifs which was PCR-amplified with primers 5′-CAGGAACAGACACCTCGCTG-3′ (forward) and 5′-GCGGCGGTCCAGGTGTCCG-3′ (reverse) differed, according to the differing number of Sp1 motifs, in length (221–253 bp). Forward primers were modified at the 5′ end with fluorescent dyes HEX, FAM or TET (MWG, Ebersberg, Germany) for detection of fragments of varying lengths by different wave length emissions after electrophoresis in the ABI prism 377 DNA Sequencer (Applied Biosystems, Foster City, USA). VNTR alleles were confirmed by DNA sequencing on an ABI 3100 sequencer (Applied Biosystems) with primers ALOX5Fseq-AAGCAATTCAGGAGAGA ACGAGT (forward) and ALOX5Rseq CTGAGGTAGATG-TAGTCGTCAGT in at least two independent sequencing reactions (reverse).

The exonic variant g.760G>A was analysed by dynamic allele-specific hybridization with fluorescence resonance energy transfer in a LightTyper device (Roche Diagnostics, Mannheim, Germany). Primer pairs for PCR amplification of a 122 bp fragment were 5′-GGAAGACCTGATGTTTGGCT A-3′ (forward primer) and 5′-GCTCCAGGCTGCACTCTA-3′ (reverse primer); sensor and anchor oligonucleotides were 5′-CAGAGCTGCCCGAGAAGC-3′ and 5′-CCCGGTGACCA CGGAGAT-3′, respectively.

*Databases and statistics*. Demographic data, self-reported signs and symptoms as documented on structured questionnaires, as well as laboratory results, were double-entered into a Fourth Dimension database (San Jose, CA, USA). Bacteriological data were provided as datasheets. Data were locked before using them in a pseudonymized form for statistical analyses.

Power calculation was performed with the CATS software (available at http://www.sph.umich.edu/csg/abecasis/CaTS/). Multivariate logistic regression analyses were calculated to determine ORs for genotype distributions (STATA 8.2 software; Stata Corporation, College Station, TX, USA). As age, sex and ethnicity significantly influenced one or more of the parameters studied, all calculations included appropriate adjustments for these variables. Analyses to calculate allele distributions and Hardy–Weinberg equilibria were performed with a public STATA module (www-gene.cimr.cam.ac.uk/clayton/software/stata/genassoc; David Clayton, Cambridge, UK). Haplotypes were estimated with the COCAPHASE software (version 2.404; Frank Dudbridge, http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/).

Corrections for multiple testing applied (i) to the two *ALOX5* variants tested and (ii) when stratifications by the mycobacterial lineages Indo-Oceanic, East-Asian, Euro-American, West-African 1, West-African 2 and *M. bovis* (17) were made. Corrected *P*-values ($P_c$) $<0.05$ were considered significant and correction values were indicated where applicable.

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*Conflict of Interest statement*. All authors have no conflict of interests.

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