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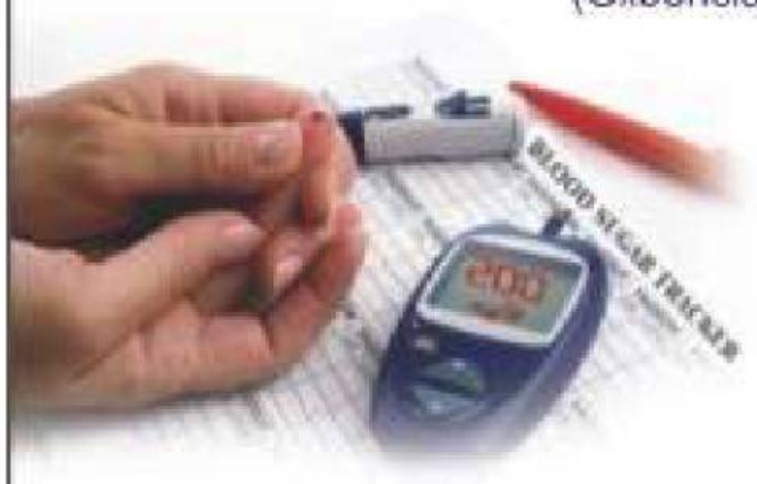

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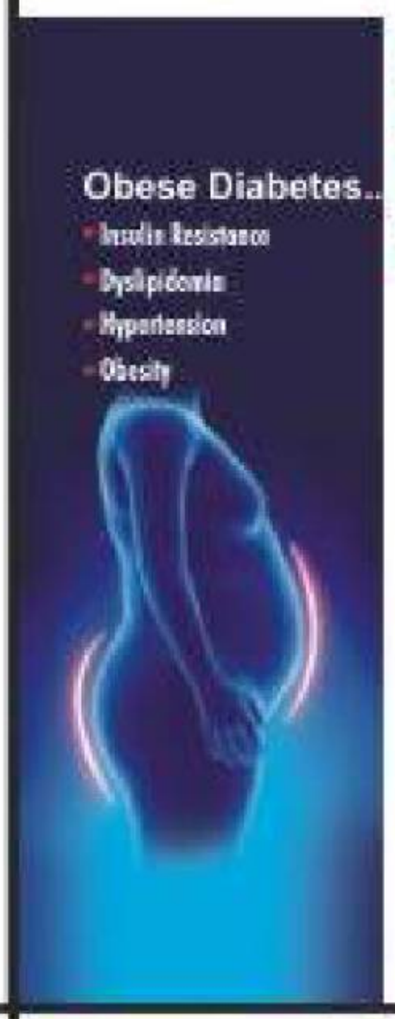

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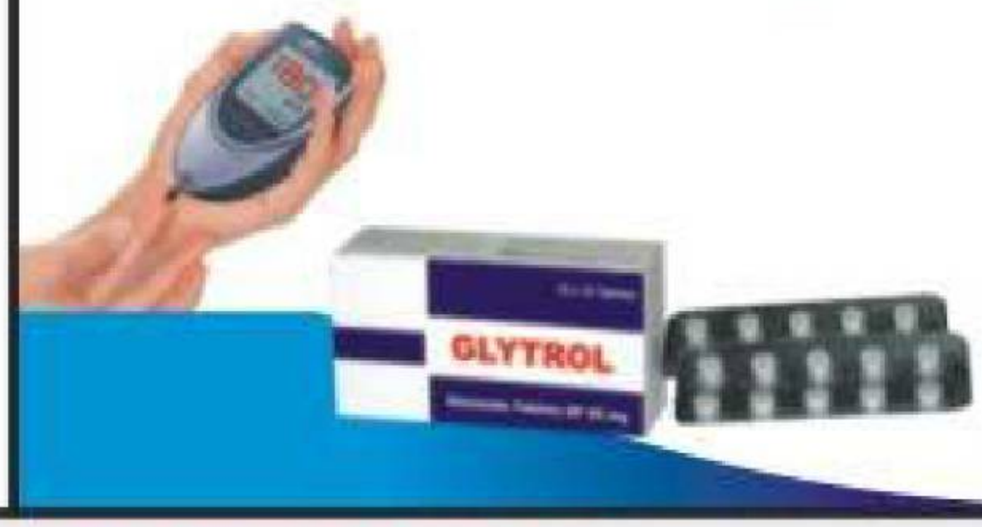

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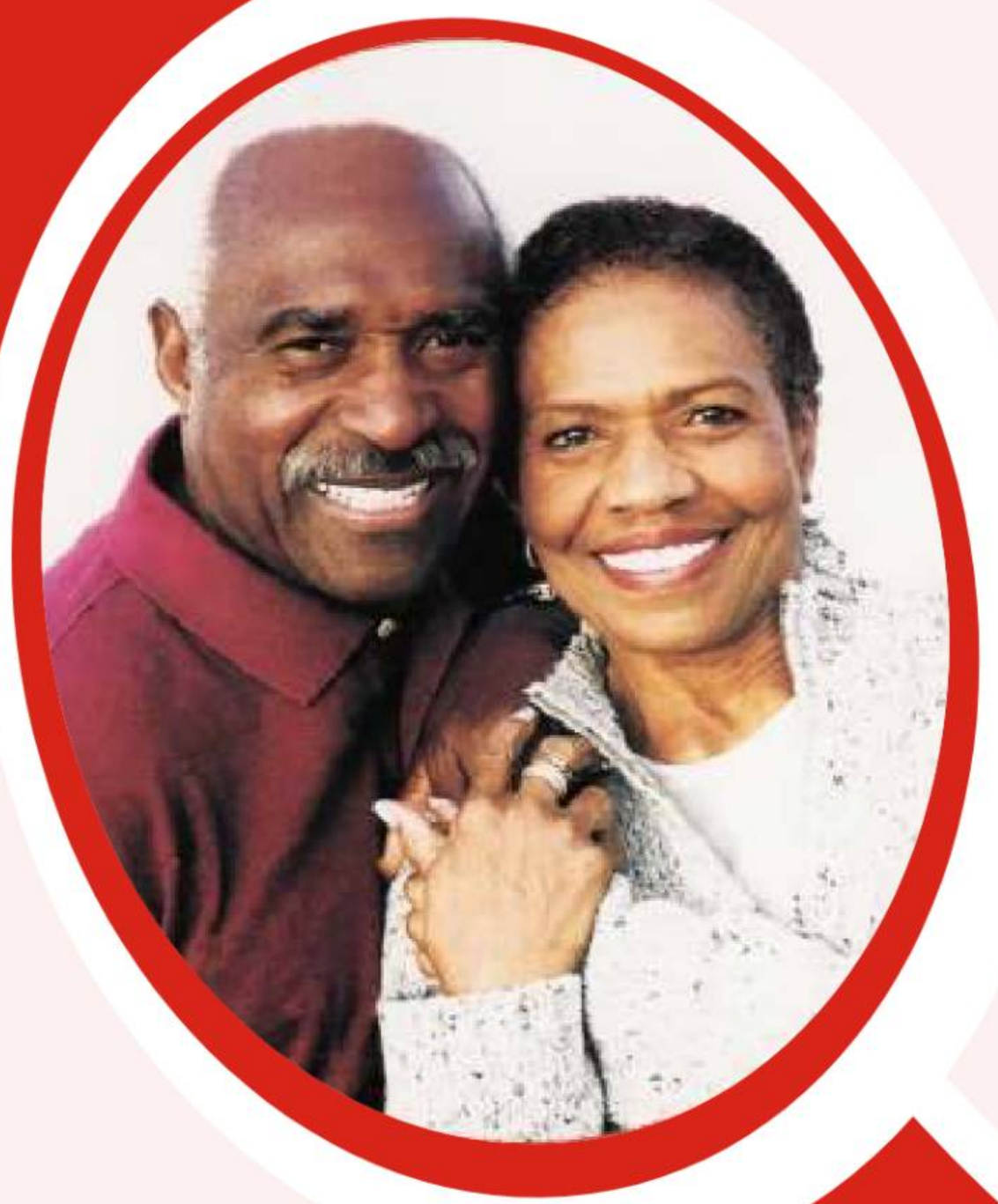

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Disease-Associated Streptococcus pneumoniae Genetic Variation

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Abstract

Streptococcus pneumoniae is an opportunistic pathogen that causes substantial illness and death among children worldwide. The genetic backgrounds of pneumococci that cause infection versus asymptomatic carriage vary substantially. To determine the evolutionary mechanisms of opportunistic pathogenicity, we conducted a genomic surveillance study in China. We collected 783 S. pneumoniae isolates from infected and asymptomatic children. By using a 2-stage genomewide association study process, we compared genomic differences between infection and carriage isolates to address genomic variation associated with pathogenicity. We identified 8 consensus k-mers associated with adherence, antimicrobial resistance, and immune modulation, which were unevenly distributed in the infection isolates. Classification accuracy of the best k-mer predictor for S. pneumoniae infection was good, giving a simple target for predicting pathogenic isolates. Our findings suggest that S. pneumoniae pathogenicity is complex and multifactorial, and we provide genetic evidence for precise targeted interventions.

Streptococcus pneumoniae is a pathogen that causes community-associated infections in young children <5 years of age [1,2]. It can asymptomatically colonize the nasopharynx and upper airway in healthy children (up to 60%) and can also invade sterile sites and lead to infections from mild to life-threatening, which can result in substantial illness and death worldwide [1,3,4]. Despite the widespread use of pneumococcal vaccines to immunize children, S. pneumoniae remains the leading cause of life-threatening diseases. Worldwide, the increasing disease burden of S. pneumoniae is alarming; an estimated 1 million children <5 years of age die of pneumococcal disease every year [5]. All pneumococcal diseases arise from bacterial colonization, and the adaptability of the virulence characteristics enhances pneumococcal persistence in colonization of the host respiratory tract, suggesting that nasopharyngeal carriage of S. pneumoniae plays a key role in development and transmission of pneumococcal diseases [6]. Pneumococcal disease is one of the most common infectious diseases caused by asymptomatic S. pneumoniae colonization in humans. Eliminating this opportunistic pathogenic bacterium requires knowledge of the pathogenicity-associated genetic elements that distinguish infection from carriage isolates. Previous studies have been limited to exploring virulence factors and molecular characterization of invasive S. pneumoniae isolates [7,8].

Whole-genome sequencing (WGS) has become a powerful tool for bacterial genotyping; costs have been decreasing as accessibility increases. The high-dimensional genomic data can provide unprecedented resolution for identifying subtle genomic variations. Genomewide association studies (GWAS) are increasingly used to detect novel genes and genetic elements associated with bacterial phenotypes, which may provide insight for future preventive strategies and control measures [9–12]. In brief, traditional GWAS methods can be used to identify large numbers of common genetic variants, usually single-nucleotide polymorphisms (SNPs), to determine the genetic basis of bacterial phenotypes of interest. However, considering the high genomic plasticity of many species of bacteria, traditional GWAS methods can only partially identify the phenotype-associated genetic variants. To avoid the limitations of SNP-based GWAS, we used k-mers (DNA words of length k) as an alternative method, which can capture different types of variants [13,14].

To determine whether genetic

variation is unevenly enriched in S. pneumoniae infection isolates, we used multiple GWAS analyses to compare genomic differences between infection and carriage isolates. Study protocols were approved by the Ethics Committee of Guangdong Pharmaceutical University (2019–19) and the Ethics Committee of Liuzhou Maternity and Child Healthcare Hospital (2018–84). We obtained written informed consent from parents or legal guardians on behalf of the children.

Methods

Sampling

During 2015–2021, we collected clinical samples from infected children and nasal swab samples from healthy children in southern China (Guangxi and Guangdong Provinces). From hospitalized infected children, we collected 349 nonrepetitive pneumococcal isolates (e.g., blood, bronchoalveolar lavage fluid, sputum, middle ear fluid), of which 342 were noninvasive and 7 invasive. The eligibility criteria for infected children were having clinical infectious manifestations such as cough, respiratory secretions, abnormal lung sounds, dyspnea, or fever >38°C, with or without infiltrates seen on chest radiographs; having *S. pneumoniae* infection diagnosed by clinical doctors on the basis of signs and symptoms; and having *S. pneumoniae* isolated from clinical infection sites. In terms of asymptomatic carriage isolates, we sampled 434 isolates from healthy children in kindergarten.

Whole-Genome Sequencing

We performed high-throughput genome sequencing on a Hiseq 2000 machine (Illumina, https://www.illumina.comExternal Link) to obtain paired-end 150-bp reads. We assessed the quality of the raw sequenced reads by using FastQC version 0.11.5 (https://github.com/s-andrews/FastQCExternal Link) and trimmed for low quality reads and adaptor regions by using Trimmomatic version 0.36 (https://github.com/usadellab/TrimmomaticExternal Link). We then assembled trimmed reads by using SPAdes version 3.6.1 (https://github.com/ablab/spadesExternal Link). We used PathogenWatch (https://pathogen.watch) to predict global pneumococcal sequencing cluster (GPSC), multilocus sequence typing (MLST), and serotyping for all genomes.

Phylogenetic Analyses

To generate the variant sites with SNPs, we mapped assembled contigs to a standard reference genome S. pneumoniae R6 by using Snippy version 4.4.5 (https://github.com/tseemann/snippyExternal Link). We used the generated core SNP alignment to construct a maximum-likelihood phylogenetic tree by using the generalized time reversible plus gamma model and 100 bootstrap replicates with FastTree version 2.1.10 (http://www.microbes online.org/fasttreeExternal Link). We visualized and annotated the phylogenetic tree by using ChiPlot (https://www. chiplot.online).

Counting and Annotating k-mers

We scanned all k-mers that were 9- to 100-bp long from all assembled reads by using fsm-lite (https://github.com/nvalimak/fsm-lite External Link) and filtered them to obtain 10,591,337 k-mers seen on 1%–99% of the total samples. To identify the relevant genes by using BWA-MEM (the Burrows-Wheeler Aligner with maximal exact matches alignment tool, https://github. com/lh3/bwaExternal Link), we mapped all k-mers to 10 S. pneumoniae reference genomes (CGSP14, D39, Hungary19A-6, R6, Taiwan19F-14, TIGR4, Spain23F-ST81, ATCC 49619, EF3030, and MDRSPN001) obtained from the Virulence Factor Database (http://www.mgc.ac.cn/VFsExternal Link) and previous studies. We determined gene ontology annotations by using the UniProt (https://beta.uniprot.orgExternal Link).

Multiple GWAS Analyses of Disease-Associated k-mers

To explore the genome wide associations between genetic elements (k-mers) and *S. pneumoniae* disease status (infection or carriage), and thus to identify infection-associated k-mers, we used GWAS methods. Because of the high-dimensional genomic data structures, we used multiple GWAS methods: the linear mixed model (LMM; (https://github.com/mgalardini/pyseerExternal Link), phylogenetic-based approach (Scoary; https://github.com/AdmiralenOla/Scoary External Link), variable selection using random forests (VSURF; https://github.com/robingenuer/VSURFExternal Link), and least absolute shrinkage and selection operator (LASSO; https://scikit-learn.org/stable/modules/generated/sklearn.linear_model.Lasso.htmlExternal Link) regression.

In brief, we used a 2-stage analysis process to detect the infection-associated k-mers by comprehensive GWAS analyses (Figure 1). First, we fitted a univariate LMM to initially screen infection-associated k-mers by using the Pyseer tool (version 1.3.10) [15]. To correct for the population structure, we used the similarity pyseer command of Pyseer, which computes a similarity kinship matrix on the basis of the core genome SNPs. For covariates, the GWAS analysis used host age (years) and sex. Second, we used multiple methods (Scoary, LASSO, and VSURF) to minimize false-positive associations and identify consensus infection-

associated k-mers by Venn diagram. In the GWAS analyses, we used the Bonferroni correction (α/N) to control for false-positive rates resulting from multiple comparisons of 1,418,815 k-mers (adjusted p value threshold 3.52×10^{-8}). Scoary is an ultrafast software tool for GWAS analyses that uses a phylogenetic-based method to adjust population structure. The LASSO regression is suitable for high-dimensional data structures, and the coefficients of nonrelevant variables can be compressed to zero to solve the problem of model overfitting [16]. We used VSURF, based on random forest (RF), to perform a 2-step feature selection on the variables [17]. Initially, VSURF ranks the variables according to the importance measure by using the RF permutation-based score of importance to obtain a subset of important variables, and then it uses a stepwise forward strategy for variable introduction based on the smallest out-of-bag error. More precisely, a variable is added only if the error decrease is larger than a threshold. We ranked the importance of k-mers by the mean decrease in impurity (mean decrease Gini), which is a measure of the predictor's contribution to the correct sample classification. We compiled associated phenotype data for all 783 isolates (Appendix Table 1) and deposited sequences in the National Center for Biotechnology Information Sequence Read Archive database (https://www.ncbi.nlm.nih.gov/sraExternal Link; projection no. PRJNA976286). The k-mer sequences and output results files from several GWAS analyses are publicly available (https://doi.org/10.6084/m9.figshare.24466606.v3External Link).

Figure 1

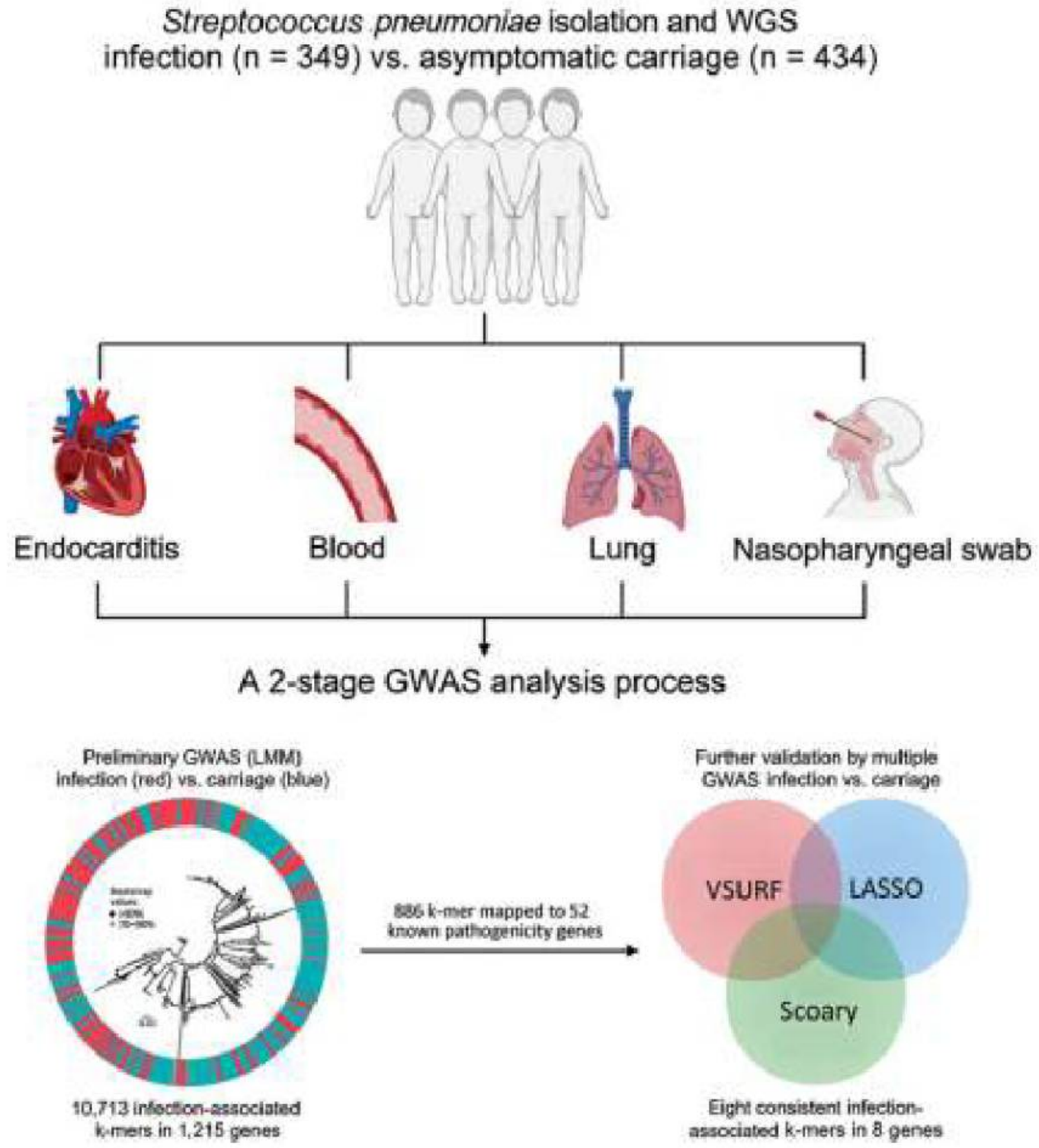

Figure 1. Two-stage GWAS analysis process used to detect infection-associated *Streptococcus pneumoniae* k-mers in study of disease-associated *Streptococcus pneumoniae* genetic variation. GWAS, genome-wide association studies; LASSO, least absolute shrinkage and selection operator; LMM, linear mixed model; VSURF, variable selection using random forests; WGS, whole-genome sequencing.

Results

Characteristics of *S. pneumoniae* Isolates

Of the 349 children with *S. pneumoniae* infection, 342 (98.0%) had noninvasive disease (264 pneumonia, 49 bronchitis, 13 otitis media, 9 upper respiratory infection, 6 nasosinusitis, and 1 corneal ulcer), and 7 (2.0%) had invasive disease (6 bacteremia and 1 endocarditis). χ^2 test results indicated no differences between infection and carriage isolates with regard to host sex (p = 0.359) but significant differences with regard to age (p<0.001) (Appendix Table 2).

Association between Genotypes and Disease Status

The most prevalent GPSCs for infection isolates were GPSC1 (45.9%), GPSC321 (9.2%), and GPSC852 (5.4%); the predominant GPSCs for carriage isolates were GPSC321 (16.1%), GPSC1 (15.4%), and GPSC23 (15.0%). In terms of sequence types (STs), the most common genotypes for infection isolates were ST271 (29.2%), ST320 (9.5%), and ST902 (7.2%); the predominant genotypes for carriage isolates were ST902 (15.9%), ST90 (13.8%), and ST271 (8.5%). The most prevalent

Figure 2

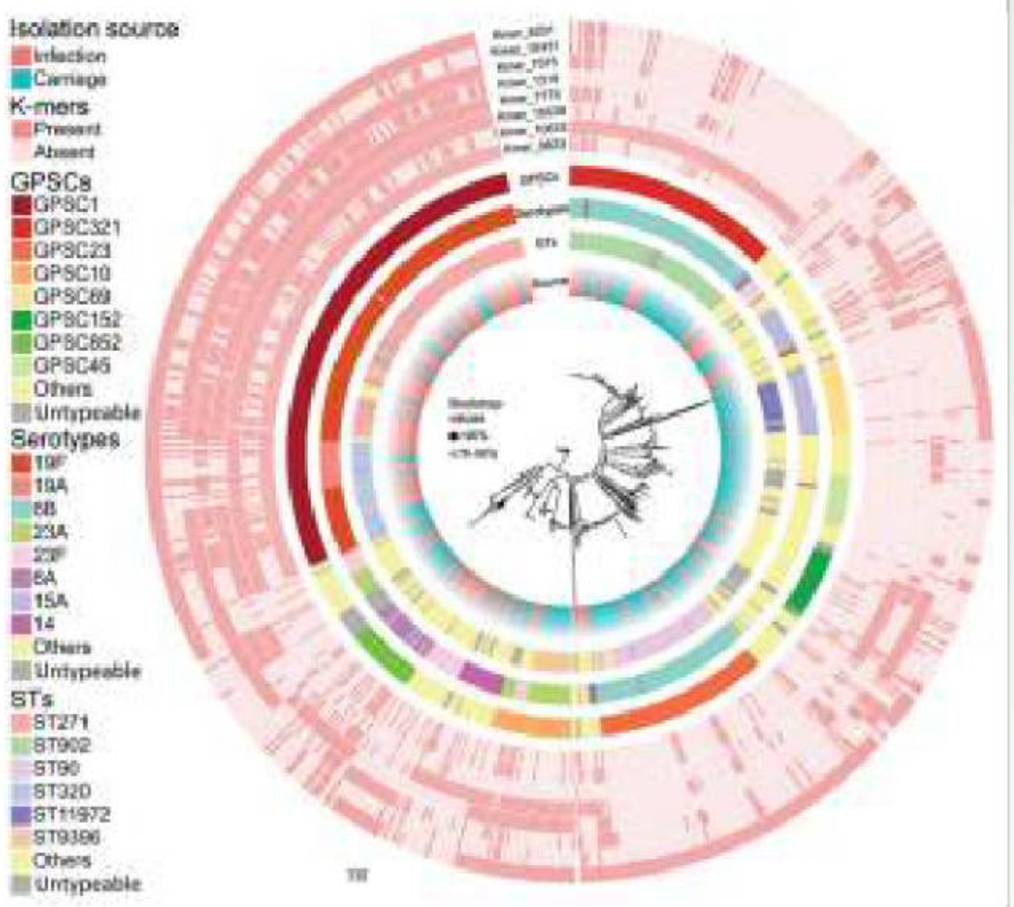

Figure 2. Whole-genome phylogenetic tree showing genetic similarity of 783 *Streptococcus pneumoniae* isolates in a study of disease-associated *Streptococcus pneumoniae* genetic variation. The colored strips at the tips of the tree (from inner to outer) represent isolate metadata (source, STs, serotypes, and GPSCs) and infection-associated k-mers found in the final model. GPSC, global pneumococcal sequencing cluster; ST, sequence type.

Figure 3

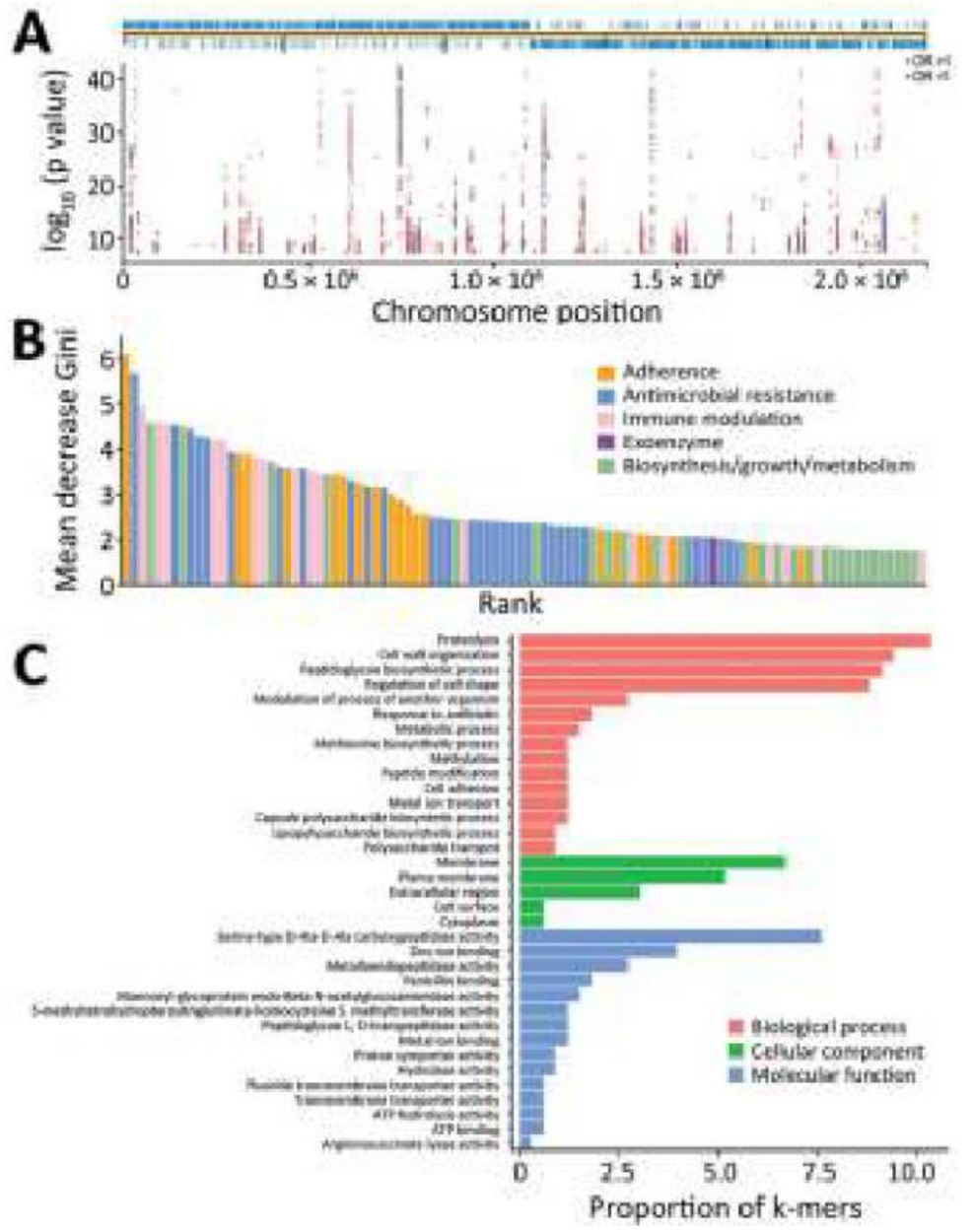

Figure 3. Preliminary screening for infection-associated k-mers by linear mixed model in study of disease-associated Streptococcus pneumoniae genetic variation. A) Manhattan plot showing statistical significance and chromosomal location of k-mers mapped to a complete reference genome (TIGR4; GenBank accession no. NC_003028.3). B) Importance of the top 100 k-mer predictors in a simpler model with 886 k-mers. C) Gene ontology annotations of the top 100 k-mer predictors. OR, odds ratio.

serotypes for infection isolates were 19F (43.0%), 6B (15.2%), and 23F (8.3%); and the predominant serotypes for carriage isolates were 6B (32.7%), 19F (13.1%), and 15A (11.1%). The results indicated potential genotype differences between infection and carriage isolates. In addition, the phylogenetic tree based on core SNPs revealed that several genotypes (GPSCs/STs /serotypes) from infection and carriage isolates clustered in the same branches (Figure 2). Moreover, we found statistically significant differences in the proportion of specific GPSCs/STs/serotypes between infection and carriage isolates (Table 1), indicating that these isolates are associated with infection.

Preliminary Screening for Infection-Associated k-mers by LMM

We identified 10,591,337 k-mers from the assemblies of 783 *S. pneumoniae* isolates and then filtered out low-frequency k-mers for a reduced matrix with 1,418, 815 k-mers. Using those k-mers for GWAS, we performed a univariate LMM analysis to initially identify 22,790 infection-associated k-mers; 10,713 k-mers were successfully mapped to 1,215 unique genes (Figure 3, panel A; Appendix Figure 1). In the initial model with 10,713 k-mers, we used the RF model to assess the prediction effect. The classification balanced accuracy based on cross-validation was 93.60% (95% CI 91.48%–95.72%) (Table 2); the area under the curve (AUC), based on the out-of-bag risk scores of the classifier, was 0.98. In the LMM analysis, the QQ-plot indicated that population structure was well controlled at low p values ($p<0.01$) (Appendix Figure 2). Because of the considerable redundancy among the genetic elements in risk prediction, studying all k-mer combinations

had little benefit; therefore, we used a simpler model with 886 k-mers successfully mapped to 52 antibiotic resistance or virulence genes (Appendix Table 3). The classification balanced accuracy was 91.28% (95% CI 89.34%–93.22%) (Table 2); the AUC was 0.96, suggesting that the power of these 886 k-mers for predicting disease status was close to that of the model with 10,713 k-mers.

In addition, we sorted the 886 disease-associated k-mers according to estimated importance (Figure 3, panel B). The k-mers were mainly associated with antimicrobial resistance (34%), adherence (20%), immune modulation (17%), and exoenzyme (1%). Moreover, the k-mers were divided into 3 functional gene ontology categories. Among those categories, proteolysis and cell wall organization were the largest subcategories in the biological process, membrane was the most enriched term in the cellular component, and serine-type D-Ala-D-Ala carboxypeptidase activity was the top term in the molecular function (Figure 3, panel C).

Figure 4

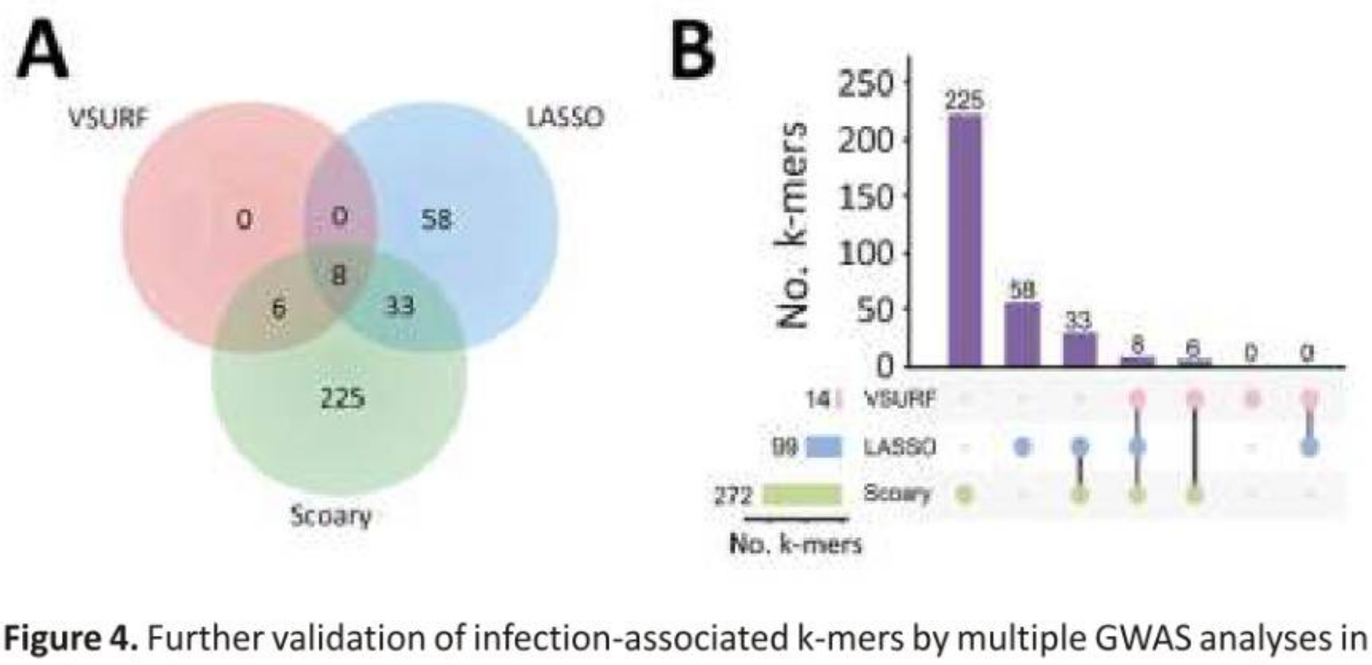

Figure 4. Further validation of infection-associated k-mers by multiple GWAS analyses in study of disease-associated Streptococcus pneumoniae genetic variation. A) Venn diagram visualization of the k-mers identified by 3 methods. B) UpSet plot visualization of the k-mers identified by 3 methods. LASSO, least absolute shrinkage and selection operator; VSURF, variable selection using random forests.

Figure 5

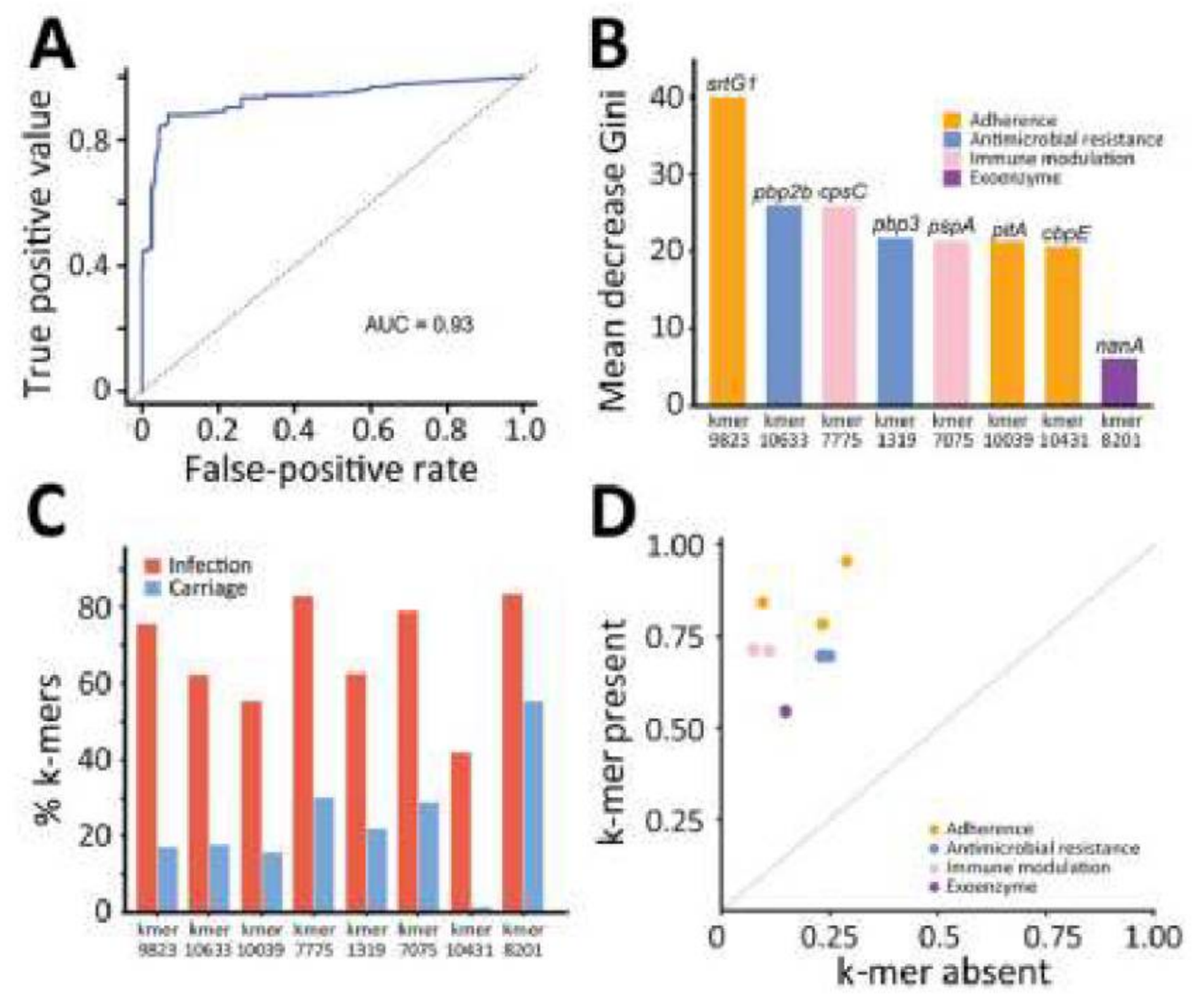

Figure 5. Prediction effect of the 8 k-mers identified in the final model used in study of disease-associated Streptococcus pneumoniae genetic variation. A) Receiver operating characteristic curve of the final model. B) Predictor importance of the 8 k-mers in the final model. C) Proportion of k-mer predictors between infection and carriage isolates. D) Change in risk score for a specific k-mer profile when the k-mer is present (y-axis) compared to absent (x-axis). AUC, area under the curve.

Further Validation of Infection-Associated k-mers by Multiple GWAS Analyses

To reduce the complexity of the model, we used 3 methods to identify consensus infection-associated k-mers (Figure 4). On the basis of the 886 k-mers screened above, we observed consensus on genomewide statistically significant associations for pathogenicity k-mers; 8 k-mers were identified by all 3 methods. When we used the simplest model with the 8 k-mers, the classification balanced accuracy was 90.89% (95% CI 89.48%–92.31%) (Table 2), and the AUC value was 0.93 (Figure 5, panel A), suggesting that the power of the 8 k-mers to predict disease status was comparable to that of the model with 886 k-mers. Of note, the k-mer predictors still exhibited high classification balanced accuracy in the predominant GPSCs (95.34% for GPSC1 and 92.79% for GPSC321). The importance of the selected k-mers in the final model indicated that these predictors were mainly associated with adherence function (Figure 5, panel B). The highest ranked predictor (Kmer_9823 in sortase

[srtG1]) achieved a classification accuracy of 79.57% on its own and also showed high classification accuracy in the predominant GPSCs (70.04% for GPSC1 and 85.29% for GPSC321). In addition, the best predictor (in srtG1) was associated with GPSC1 and GPSC321 (all $p<0.05$). For the additional validation analysis that used the best RF classifier k-mer (in srtG1), 2 independent datasets of *S. pneumoniae* genomes with genotype distribution similar to that of our study were available on the National Center for Biotechnology Information Assembly database (https://www.ncbi.nlm.nih.gov/assemblyExternal Link (data1: 60 noninvasive vs. 60 carriage isolates; data2: 60 invasive versus 60 carriage isolates; the prevalence of the predominant GPSCs [GPSC1 and GPSC321] was 58.3% for noninvasive, 55.0% for invasive and 30.0% for carriage isolates) (Appendix Table 4). Classification accuracy was 75.83% for data1 and 74.17% for data2, similar to that in the larger primary dataset in our study.

The proportion of k-mers differed significantly between infection and carriage isolates (all $p<0.05$) (Figure 5, panel C), indicating that the proportion of k-mers was substantially higher in infection isolates than in carriage isolates. The effect of each k-mer on the estimated risk score (Figure 5, panel D), indicated by a point above the diagonal, indicates that the risk score is increased when the k-mer profile is present. The presence of k-mers associated with adherence genes markedly increased the risk for S. pneumoniae infection (odds ratio [OR] 1.88 for Kmer_9823, OR 1.65 for Kmer_10039, and OR 1.69 for Kmer_10431) (Table 3).

Discussion

To explore genomic differences between infection and carriage isolates, linking infection-associated genotypes with disease status is necessary. In our study, the most common serotypes for infection isolates (19F, 6B, 23F) were consistent with the results from other regions of China [18–20] but differed from those from the United States and Japan [21,22]. Moreover, we observed considerable ST diversity among infection isolates; the most prevalent genotypes were ST271, ST320, and ST902, a finding consistent with those of previous studies in China but different from those in developed and developing countries [23–25]. The resolution of MLST and serotyping for inferring isolate relatedness is limited, so we also used GPSCs to characterize and compare different lineages [26]. The most prevalent GPSCs among the infection isolates were GPSC1, GPSC321, and GPSC852, which differed from those in the United States and South Africa [27]. Our findings suggest that discrepancy in genotypes on a global scale may be associated with different pathogenicity and evolutionary directions. In our study, associations between specific genotypes (such as 19F and GPSC1) and disease status differed significantly, which is consistent with findings from a study in India [28]. Our findings indicate that the presence of specific pathogenic clones may promote infection. In a simple pathogenicity model, all pathogenic clones would belong to specific clusters of genetically related disease-causing isolates (i.e., pathogenic clone hypothesis; Figure 6, panel A), which has been observed for Staphylococcus aureus and S. pneumoniae isolates [29,30]. That pathogenicity model is not suitable for all S. pneumoniae clones because many infection isolates clustered in the same branches of phylogenetic tree as carriage isolates. In addition, traditional genotypes provide little power for identifying small genetic variations at the genomic level [29], suggesting that those genotypes only partially explain the pathogenicity of *S. pneumoniae*.

Using high-throughput genome sequencing technologies and bacterial GWAS methods to further explore high-dimensional genetic variation between infection and carriage isolates is essential, thereby revealing the pathogenicity-associated genetic elements of *S. pneumoniae*. According to the phylogenetic tree, we observed that infection isolates were markedly unevenly distributed across the phylogeny and also clustered

Figure 6

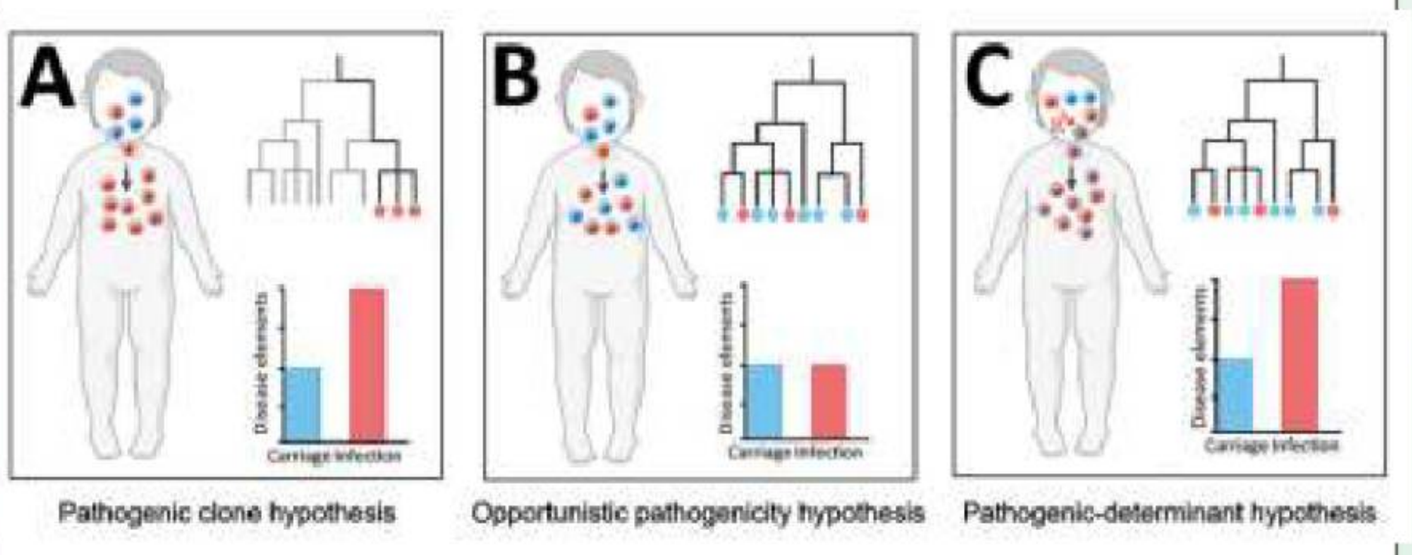

Figure 6. Pathogenicity models for genetically related disease-causing isolates used in study of disease-associated *Streptococcus pneumoniae* genetic variation. A) Pathogenic clone hypothesis; B) opportunistic pathogenicity hypothesis; C) pathogenic-determinant hypothesis.

with carriage isolates within several lineages, indicating that most lineages are capable of causing infection (i.e., opportunistic pathogenicity hypothesis; Figure 6, panel B). If this hypothesis is reasonable, then GWAS analyses would not detect numerous pathogenicity-associated k-mers. However, the LMM-based GWAS in our study detected 22,790 pathogenicity-associated k-mers. These findings suggest that the enrichment of genetic elements encoding pathogenicity traits may increase the pathogenicity of *S. pneumoniae* (i.e., pathogenic-determinant hypothesis; Figure 6, panel C), which is consistent with *Staphylococcus epidermidis* and avian pathogenic *Escherichia coli* [30,31]. In this pathogenic-determinant model, horizontal gene transfer could spread genetic determinants in bacteria such as *S. pneumoniae* and *Klebsiella pneumoniae* [32–34], leading various clones to successfully cause disease.

High-throughput genomic data have brought substantial challenges to data analysis because of high-dimensional and highly correlated data structures. In our study, we identified infection-associated k-mers by using a 2-stage comprehensive GWAS analysis process, including LMM for initially screening pathogenic k-mers and multiple GWAS methods for further validation. In the final prediction model, we identified 8 k-mer predictors, which mapped to genes associated with adherence, immune regulation, antibiotic resistance, and exoenzyme. Of the adherence-related genes, *srtG1* and the LPxTG-type surface-anchored protein (*pitA*) are important components of the pneumococcal pilus-2, which plays a crucial role in promoting adhesion, colonization, and cellular invasion [35,36]. Classification accuracy of the most important k-mer in *srtG1* was high by itself, and that of the additional validation RF analysis based on open datasets was similar, suggesting that this predictor has great potential for predicting pathogenic isolates in a clinical setting. Phosphorylcholine esterase (*cbpE*) plays an important role in modulating both the phosphorylcholine decoration of its surface and choline-bound surface adhesins, which may contribute to pneumococcal adherence and invasiveness [37]. Capsular polysaccharide (CPS) is a major virulence factor in *S. pneumoniae*. Capsular polysaccharide protein C (CpsC) has been shown to affect the level of CPS expression and also regulate the assembly, export, and attachment of CPS to the cell wall [38]. Pneumococcal surface protein A (PspA) plays role in preventing complement-mediated opsonization and is also capable of binding to lactoferrin, thereby preventing it from killing pneumococci [39]. The infection-associated genes reported in our study (*cpsC and pspA*) are homologous to the genes associated with invasive pneumococci (*cpsA, cpsD, and pspC*) identified in previous studies [11,12], providing more evidence for *S. pneumoniae* pathogenicity. Neuraminidase A encoded by the nanA gene is an essential colonization factor for *S. pneumoniae* and promotes growth and survival of the bacteria in the upper respiratory tract [40]. Antimicrobial drug use and abuse not only induce widespread multidrug-resistant pneumococci but also increase the susceptibility to invasive disease [41]. For decades, penicillin has been the first choice for treatment of pneumococcal infection, and mutations in penicillin-binding proteins (PBPs) are essential for high-level penicillin resistance [42]. Li et al. demonstrated that *pbp2b* and *pbp3* are associated with pneumococcal infection [42]. One reason is that PBP2B and PBP3 are involved in the synthesis and growth of bacterial cell walls, which are crucial for the survival and virulence of pneumococci [43]. In addition, a previous study revealed a potential association between penicillin resistance and GPSC1 [44], and our findings also showed that GPSC1 was associated with pneumococcal infection, suggesting that it cannot support a causal link between resistance and pneumococcal infection and may result from a lineage confounder. In summary, these infection-associated k-mers provide genetic evidence for revealing optimal risk factors for infection isolates, which may offer a theoretical basis for precise targeted interventions.

Acknowledgments

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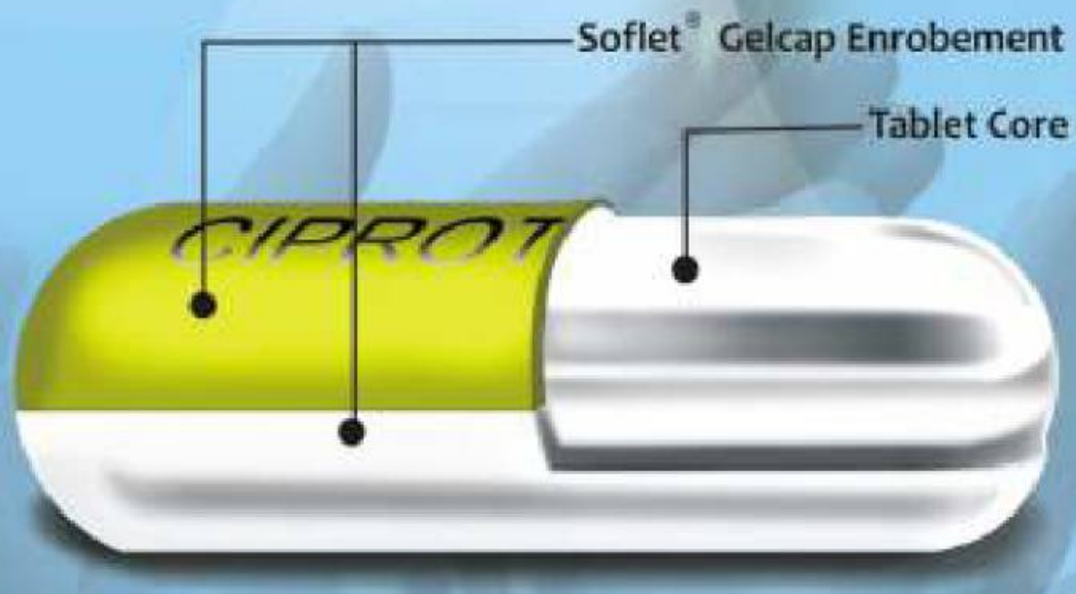

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...a smart move

Harnessing upregulated E-selectin while enhancing SDF-1α sensing redirects infused NK cells to the AML-perturbed bone marrow

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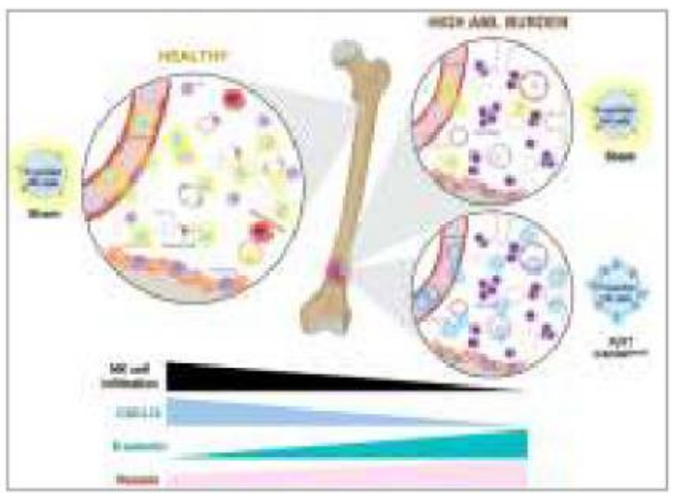
Abstract

Increased bone marrow (BM) homing of NK cells is associated with positive outcome in patients with acute myeloid leukemia (AML) treated within adoptive NK cell transfer trials. While most efforts to further improve the efficacy focus on augmenting NK cell persistence and cytotoxicity, few address their ability to home to the tumor. Here, we decipher how AML growth alters the BM niche to impair NK cell infiltration and how insights can be utilized to resolve this issue. We show that AML development gradually impairs the BM homing capacity of infused NK cells, which was tightly linked to loss of SDF-1α in this environment. AML development also triggered up-regulation of E-selectin on BM endothelial cells. Given the poor E-selectin-binding capacity of NK cells, introduction of fucosyltransferase-7 (FUT7) to the NK cells per mRNA transfection resulted in potent E-selectin binding and stronger adhesion to E-selectin+ endothelial cells. Co-introduction of FUT7 and gain-of-function CXCR4 (CXCR4R334X) redirected NK cell homing to the BM of AML-bearing mice nearly to the levels in AML-free mice. This work shows how impaired NK cell homing caused by AML-induced microenvironmental changes can be overcome by genetic engineering. We speculate our insights can help further advance future NK cell immunotherapies.



Introduction

Immunotherapy has rapidly emerged as a treatment option for a broad range of cancers. The use of chimeric antigen receptor (CAR)-T cells is currently reforming how we treat hematological malignancies such as B cell lymphomas, acute lymphoblastic B cell leukemia (ALL), and more recently also multiple myeloma (MM)[1]. Adoptive infusion of natural killer (NK) cells hold promise for the treatment of myeloid leukemias with response rates of up to 50% [2,3]. Remission in acute myeloid leukemia (AML) and high-risk myelodysplastic syndromes (MDS) have been positively linked to the bone marrow (BM) infiltration potential of the NK cells [3,4]. Yet, few studies have addressed how to redirect adoptively infused NK cells to the BM compartment [5,6]

Insights from MM and ALL have uncovered that tumor development in the BM alters the microenvironment. For instance, tumor-mediated suppression of BM stromal cells is reported to suppress SDF-1α levels in this compartment leading to poor infiltration of $CXCR4^+$ lymphocytes [7,8]. Although the SDF-1α levels in the BM can be restored following efficient tumor eradication by chemotherapy [8], this takes weeks to months and is not always possible, especially in the case of refractory disease. While reduced infiltration of adoptively infused lymphocytes has not been addressed in myeloid leukemia, it is well known that AML development triggers increased E-selectin expression on surrounding endothelial cells in the BM niche causing chemorefractoriness and AML dormancy [9,10].

Here we show that AML development in the BM suppresses SDF-1α levels and significantly

reduces the degree of infiltration by adoptively infused NK cells in a xenogeneic mouse model. We demonstrate that human NK cells electroporated with mRNA coding for fucosyltransferase-7 (FUT7) results in durable fucosylation of E-selectin ligands and thereby strong adhesion to E-selectin^{+} endothelial cells. Utilizing this insight to harness the selectively upregulated E-selectin on endothelial cells in the AML BM niche, together with overexpression of gain-of-function (GoF) CXCR4, CXCR4^{R334X}, we show that the significant reduction of NK cell BM infiltration in animals with high AML burden is almost restored to that of non-tumor-bearing animals. Overall, these results establish that AML growth in the BM suppresses the infiltration of adoptively infused NK cells via reduced levels of SDF-1α. Based on this and the known upregulation of E-selectin observed on BM vessels adjacent to the leukemic cell [9, 11,12,13], we have developed a novel approach to efficiently redirect NK cells to the AML niche, which may be explored to improve response rates of adoptive NK cell immunotherapy against myeloid leukemia.

Methods

Isolation and expansion of NK cells

Peripheral blood cells were collected from healthy blood donor buffy coats (Ethical approval Dnr 2006/229-31/3). Peripheral blood mononuclear cells (PBMCs) were isolated by high-density gradient centrifugation before being cryopreserved. NK cells were isolated from thawed PBMCs using the human NK cell isolation kit (Miltenyi, Bergisch Gladbach, Germany) for magnetic bead separation according to the manufacturer's protocol. Ex vivo expansions were performed as previously described [14]. Briefly, isolated NK cells were co-cultured with irradiated SMI-LCL feeder cells at a ratio of 1:20 in X-VIVO 20 cell culture medium (Lonza, Walkersville, MD, USA) containing 10% heat-inactivated human AB serum (Invitrogen), 2 mM GlutaMAX-1 (Gibco, Waltham, MA, USA) and 500 IU mL^{-1} IL-2 at 6.5% CO_2 and 37 °C. Fresh media was supplied to cells starting on day 4 of expansion, and then, cells were subcultured every 2–3 days until they were harvested for the experiments.

Murine xenograft tumor model

Animal experiments were performed under ethical approval (ID1533 and 22248-2022) by Jordbruksverket, Sweden. NSG-SGM3 female mice aged 2 to 5 months were used for this study. Male mice were used only when indicated. The AML mouse model was generated by intravenously (i.v.) transplanting 0.5 million HL-60 cells into NSG-SGM3 mice and the AML engraftment was evaluated in the organs by flow cytometry at different timepoints after AML cell injection. For this, tissues were mechanically dissociated, and HL-60 cells were identified by expression of human CD45 and human CD33. AML engraftment is presented as % HL-60 cells within the alive population in each tissue. For endothelial cell stains, we collected all the BM endothelial cells (BMECs) as previously described [15,16]. Briefly, bones (femur and tibia) were mechanically dissociated in PBS supplemented with 2% FBS (Gibco) and the marrow cells were collected. The cells within remaining bone fragments were also obtained following treatment with collagenase II (ThermoFisher, Waltham, MA, USA) (1 mg/mL in MEM 10% FBS) for 60–90 min under gentle agitation at 37 °C. Both cell fractions were pooled and resuspended in PBS 2% FBS before being analyzed by flow cytometry. BMECs were identified as mouse CD45^{-} LIN^{-} mouse CD31^{+} cells within the live population. Finally, in some experiments, sternums and BM supernatants were also collected for additional analyses of the AML-BM niche, using immunohistochemistry and ELISA analyses, respectively.

Immunohistochemistry (IHC) analysis

Mouse bones (sternums) were fixed in 4% paraformaldehyde (PFA) for 48 hours and decalcified in EDTA for 72 hours, before paraffin embedding and mounting 4 μm sections on slides. After deparaffinization, heat-based antigen retrieval, endogenous peroxidase blockade using 3% hydrogen peroxide, and blocking were performed. Anti-COX IV (3E11) and anti-mouse CD31 (D8V9E) rabbit monoclonal antibodies (Cell Signaling Technology, Danvers, MA, USA) were used as primary antibodies for identification of HL-60 cells and mouse endothelial cells, respectively. The primary antibodies were incubated overnight at 4 °C. Detection was performed using the Vectastain ABC-HRP Kit (Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine (DAB; Vector Laboratories). Counterstaining was performed using hematoxylin. The stained tissue sections were scanned using a 3D Histech Slide Scanner and Pannoramic MIDI (3D HISTECH) and the Pannoramic Viewer (3D HISTECH) was used for taking images of the sections at different magnifications. The substitution of the primary antibody for serum and/or staining of healthy BM (in the case of COX IV staining for tumor detection) were used as negative controls to detect specific staining.

NK cell transfection

4 µg/10^6 cells of mRNA were transfected into ex vivo expanded NK cells using the MaxCyte GT electroporation instrument (MaxCyte Inc., Gaithersburg, MD, USA). mRNAs encoding for human FUT7 were prepared from a plasmid purchased from SinoBiological (Beijing, China): using the HiScribe T7 ARCA mRNA Kit (with tailing) (New England BioLabs, Ipswich, MA, USA) and the MEGAclear™ Kit (Invitrogen) according to the manufacturer's instructions. Custom-made mRNAs encoding for human FUT7 and $CXCR4^{R334X}$ were obtained from TriLink Biotechnologies.

Cellular homing assays in vivo

Sixteen hours after FUT7 mRNA electroporation or two hours after $CXCR4^{R334X}$ mRNA electroporation, 10×10^6 NK cells were injected i.v. into healthy (tumor-free) NSG-SGM3 mice or mice with either low or high tumor burden. Animals received 2×10^5 IU of IL-2 intraperitoneally immediately after injection of the NK cells and every 24 hours. Peripheral blood, BM, spleen, liver and lungs were harvested from animals 24 or 48 hours after cell transplantation. Tissues were dissociated and the homing of NK cells were examined by flow cytometry based on human CD45 and human CD56 expression. NK cell infiltration is presented as % NK cells within the alive population in each tissue. A non-transplanted mouse was included as a negative control in each analysis.

Statistical analysis

Statistical analyses were performed using Prism (GraphPad Software Inc, San Diego, CA, USA). The Wilcoxon signed-rank test was used to assess significance in paired non-parametric datasets and the Mann-Whitney U test was used for unpaired non-parametric datasets. Significant results were marked by $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$ while non-significant results were not further specified.

Additional methods

See the Supplemental Material for additional material and methods.

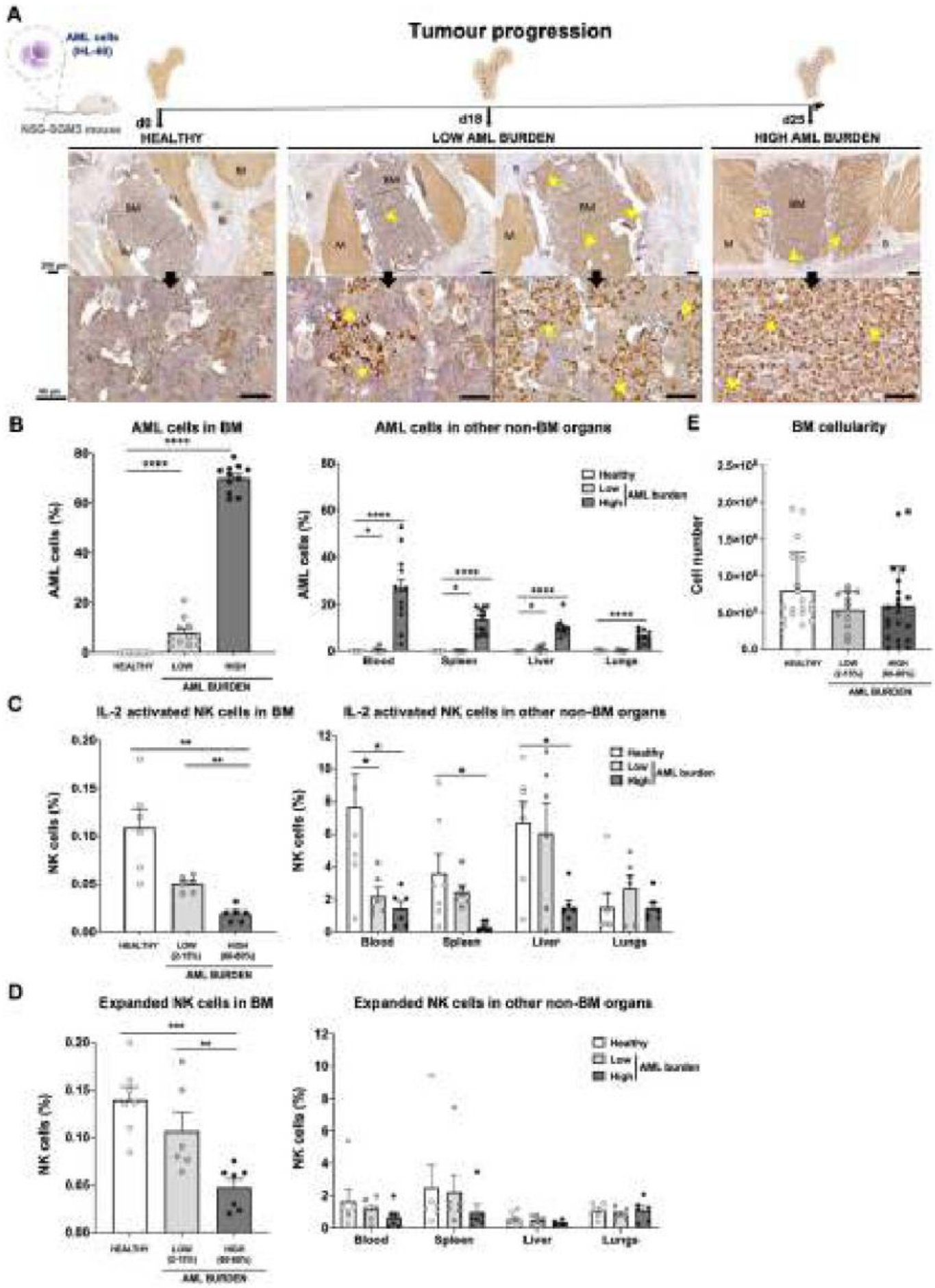
Results

Myeloid leukemia growth disrupts the BM microenvironment and impairs NK cell infiltration

To address if the development of myeloid leukemia alters the BM microenvironment and if that impairs lymphocyte infiltration, we established a robust xenograft AML mouse model. The human myeloid leukemia cell line HL-60 was chosen as it is known to establish well but grow relatively slow in NSG-SGM3 mice [17] but also because our data show it is relatively resistant to our NK cells both in vitro and in vivo (Supplemental Fig. 1), allowing us to assess NK cell homing without altering the AML burden post-NK cell infusion. As shown in Fig. 1A, B, i.v. inoculation of the HL-60 cell line into NSG-SGM3 mice resulted in low AML burden (around 2–15%) in the BM at day 18 and high AML burden (60–80%) at day 25. At the later timepoint, AML cells could also be detected in blood (26 ± 4%) and other non-BM organs such as spleen, liver, and lungs. Importantly, the animals were clinically unaffected at either of these two timepoints. Using our model, we next assessed the ability of i.v. infused human NK cells to infiltrate the BM. As shown in Fig. 1C, D, high AML burden resulted in poor BM infiltration of both IL-2 short-term activated and ex vivo expanded NK cells. Importantly, the proportion of NK cells was not influenced by changes to the total cell number in the BM as this was similar between all three conditions (Fig. 1E). The presence of NK cells in other organs such as blood, spleen and liver was also reduced when a significant amount of AML cells were present while this was not observed in the lungs that had the lowest AML burden of all organs assessed (Fig. 1C, D). These data show that AML development in the BM gradually hinders NK cell infiltration, similarly to what has been shown for lymphocytes in the context of ALL and MM [7,8].

A key factor for BM homing is the proper fucosylation of extracellular proteins (such as PSGL-1) on NK cells, which is needed for E-selectin binding and their subsequent rolling on vascular surfaces, which is one of the first steps in the leukocyte adhesion cascade [18]. Moreover, as previously reported, commonly used methods to prepare NK cells for adoptive infusion can alter the chemokine receptor expression and thereby modulate their ability to migrate toward certain chemokines in vitro [19,20]. Therefore, we next assessed the expression of key chemokine receptors and adhesion molecules on NK cells before and after ex vivo expansion. This revealed low fucosylation levels on ex vivo expanded NK cells as measured by sialyl-Lewis-X (sLe^x) expression (Fig. 2A), indicating poor binding to E-selectin. Our data also corroborate previous data [19] showing a reduction in the $CXCR4^+$ NK cell population (Fig. 2B) following ex vivo expansion. As observed in Fig. 2A, B and summarized in Fig. 2C, the altered selectin ligands and chemokine receptor expression on ex vivo expanded NK cells (Fig. 2C) collectively suggest dampened BM homing capacity.

Fig. 1: AML development disrupts the BM microenvironment and impairs NK cell infiltration.



A Experimental design of the in vivo AML model showing different stages of tumor development in the BM. Representative IHC stainings of COX IV protein in BM mouse samples from healthy (tumor-free), low and high tumor burden conditions. Arrows indicate COX IV$^+$ cells (AML blasts) present within the central BM as well as spreading to other regions. Scale bar: 200 µm (upper panels), 50 µm (bottom panels). Legend: BM (bone marrow), **B** (bone) and M (muscle). Created with BioRender.com. **B** Tumor burden in BM and several organs at different stages of AML progression after infusion of HL-60 cells into NSG-SGM3 mice, assessed by FC (n = 12 mice/group). Infiltration of **C** IL-2 activated and **D** expanded human NK cells in BM (left panel) and other organs (right panel) 24 hours after infusion comparing healthy (tumor-free), low and high tumor burden conditions, assessed by FC (n = 6–7 mice/group). Bars, mean. Error bars, SEM. **E** Total BM cell number recorded by flow cytometry after equal tissue handling and processing, comparing healthy (tumor-free), low and high tumor burden conditions. The Mann-Whitney U test comparing healthy (tumor-free), low and high tumor burden conditions was used for statistics.

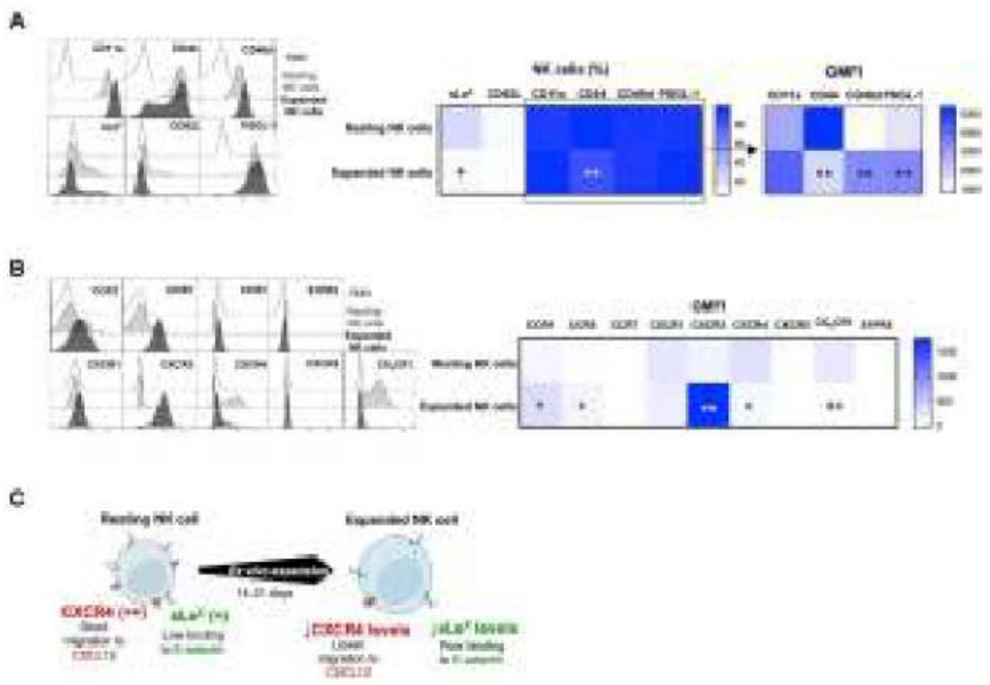
Expanded NK cells transfected to express fucosyltransferase-7 have increased sLe^x levels resulting in increased endothelial adherence under shear stress

Given the low cell surface fucosylation levels displayed by ex vivo expanded NK cells, we next wanted to address if increased levels of fucosylation would lead to better adherence to endothelial cells. This que is an early key event in the extravasation cascade [18]. Therefore, we transiently introduced FUT7, the enzyme responsible for synthesizing sLe^X, to expanded NK cells per mRNA transfection (Fig. 3A). This rapidly increased sLe^X on almost all cells for at least one week without impacting viability or the expression of key adhesion molecules (Fig. 3B, C and Supplemental Fig. 2). When challenged with shear stress in flow chamber assays, FUT7 transfected NK cells showed increased adhesion to a monolayer of TNF-α-activated HUVEC cells (Fig. 3D). Importantly, this modification did not alter NK cell function as measured by degranulation and killing of myeloid leukemia and EBV-LCL cell lines (Fig. 3E, F).

FUT7-modified expanded NK cells distribute differently in vivo compared to control NK cells in tumor-free mice with a trend for more NK cells in the BM

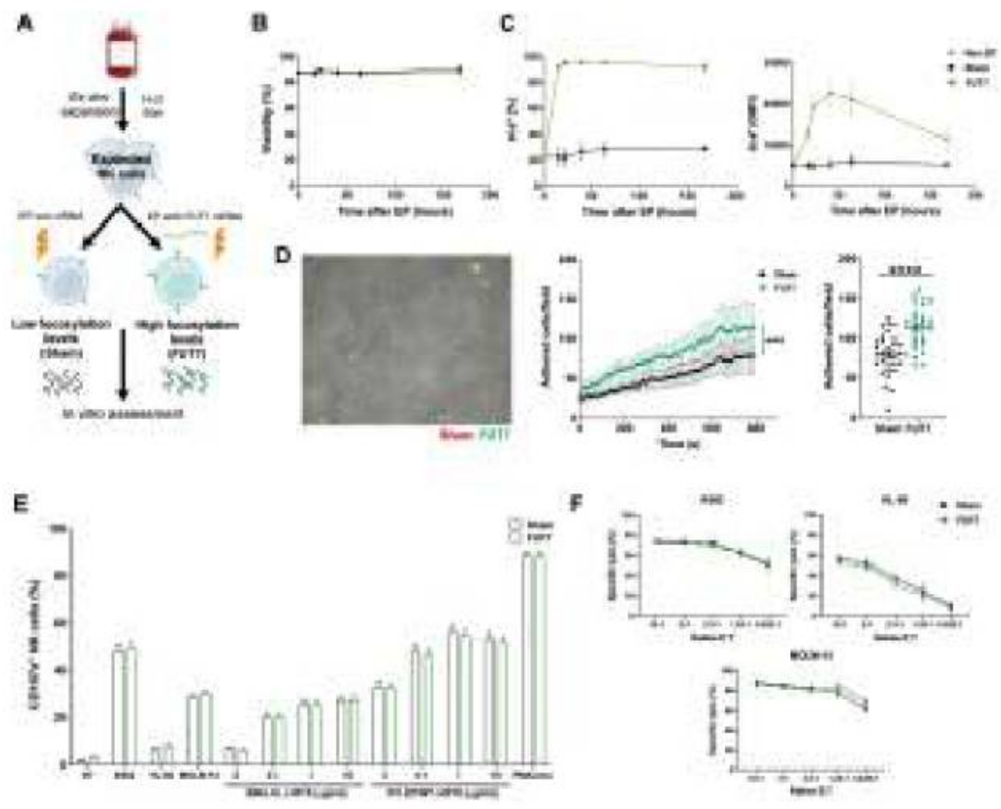
In contrast to settings of stress such as induced during tumor development, E-selectin is constitutively lowly expressed on selected endothelial cells in the body, including in the BM [21,22]. We therefore first wanted to explore if FUT7-modified NK cells had an increased potential to home to the BM of healthy tumor-free mice (Fig. 4A), similar to as shown for ex vivo fucosylated (exofucosylated) CAR T cells [23]. Following i.v. injection

Fig. 2: Expanded human NK cells have limited BM homing due to low CXCR4 and sLeX expression.



Representative histograms of relevant (**A**) adhesion molecules and (**B**) chemokine receptors on human NK cells before and after expansion and the corresponding heat maps of protein expression (NK cells (%) and/or GMFI) assessed by FC (n = 6). **C** Schematic representation showing the limited BM homing potential of NK cells after expansion. The Wilcoxon matched-pairs signed-rank test comparing human NK cells before and after expansion was used for statistics. Created with BioRender.com.

Fig. 3: FUT7+ expanded human NK cells present high fucosylation levels, increased adhesion to TNFα-stimulated HUVECs under shear stress and maintain their viability and cytotoxic potential.



A Experimental layout for electroporation of expanded human NK cells with mRNA coding for FUT7. Created with BioRender.com. **B** Viability of ex vivo expanded NK cells from healthy donors following electroporation with 4 µg of FUT7 mRNA per million cells, and C sLe^X expression levels (% and GMFI) measured by HECA-452 binding. Sham (no mRNA) (n = 12). **D** Representative capture of a flow chamber assay movie showing sham (red) and FUT7$^+$ (green) NK cells adhered to the TNFα-stimulated HUVEC under physiological shear stress and their in vitro adhesion comparing sham (non-mRNA) and FUT7 mRNA electroporated expanded NK cells (n = 6). **E** NK cell degranulation and **F** target cell killing following co-cultures with NK cells and the denoted target or without target (no target, NT) performed 20-24 hours after electroporation of the NK cells with FUT7 mRNA (n = 4–6). Bars, mean. Error bars, SEM. The Wilcoxon matched-pairs signed-rank test comparing mRNA electroporated vs sham electroporated NK cells was used for statistics.

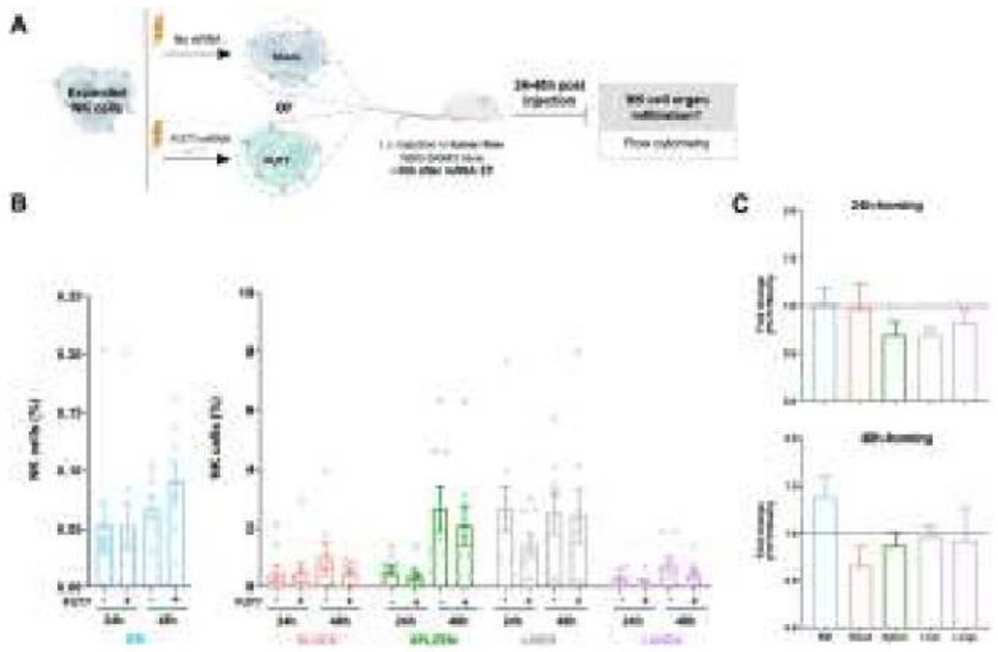
of FUT7-modified NK cells into healthy tumor-free NSG-SGM3 mice, we observed an altered in vivo distribution over time compared to control NK cells (Fig. 4B, C). Although there was a trend for more FUT7-engineered NK cells in the BM 48 hours after injection compared to control, this was not statistically significant. In fact, increased BM homing of FUT7-engineered NK cells was not always the case and highly varied between individual mice and donors (Fig. 4C), likely due to factors such as different basal E-selectin levels on the BMECs and different basal fucosylation levels of each donor. Hence, this data did not clearly indicate that FUT7-modified NK cells have a higher propensity for BM homing compared to controls when infused into tumor-free mice.

We speculate that this observation is linked to the generally low E-selectin levels on the endothelial cells in the BM under homeostatic conditions.

AML progression gradually increases vascularization and E-selectin expression in the BM resulting in increased infiltration of FUT7-modified expanded NK cells

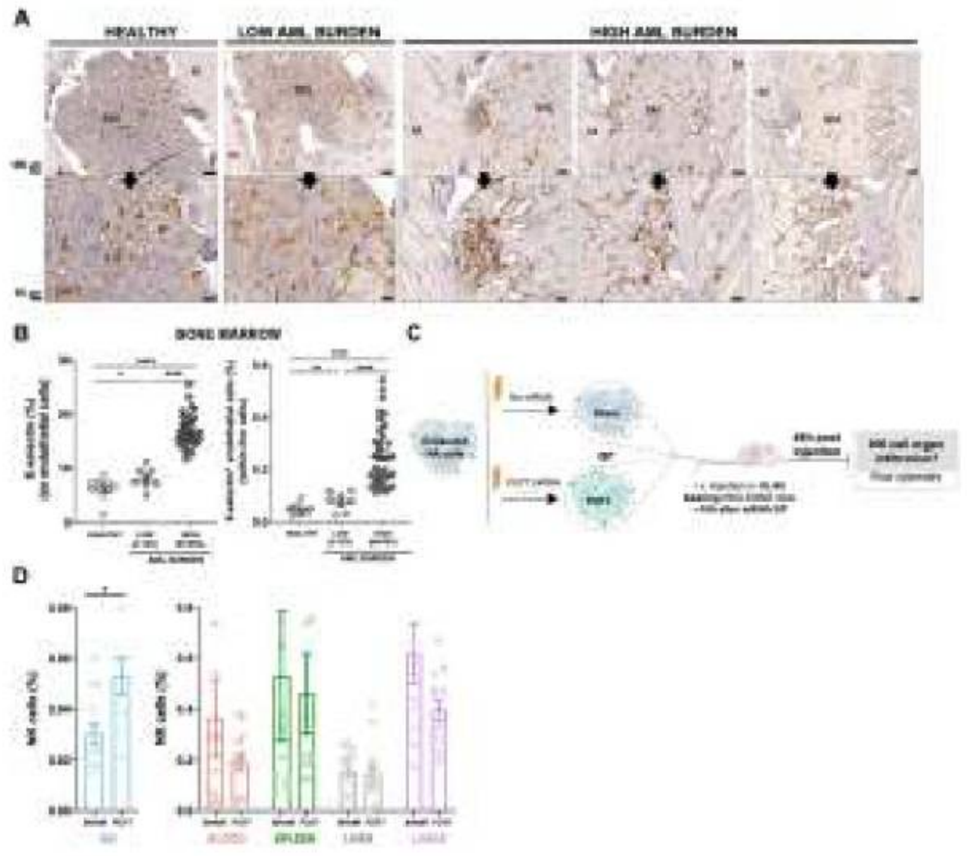
AML cells are reported to stimulate endothelial cells to upregulate E-selectin and via this interplay become dormant and chemorefractory [9,10]. Hence, as E-selectin levels on BM endothelial cells are reported to increase during AML development [11], we next assessed if this was also observed in our model. As shown in Fig. 5A, B and Supplemental Fig. 3, engraftment of HL-60 in NSG-SMG3 mice resulted in increased vascularization and E-selectin

Fig. 4: Introduction of FUT7 in expanded human NK cells does not provide a significant benefit in BM homing in tumor-free mice.



A Experimental layout for in vivo homing of adoptively infused expanded human NK cells electroporated with mRNA coding for FUT7 into healthy NSG-SGM3 mice. Created with BioRender.com. B NK cell infiltration (%) in several organs 24 h and 48 h after NK cell transfer, assessed by FC (n = 9/group). C Comparison of NK cell infiltration in several organs in terms of fold change relative to sham (no mRNA) condition. Bars, mean. Error bars, SEM. The Mann-Whitney U test comparing mRNA electroporated vs sham electroporated NK cells at each timepoint (24 h or 48 h) was used for statistics.

Fig. 5: AML progression promotes angiogenesis and E-selectin upregulation on BM endothelial cells, creating a more favorable microenvironment for FUT7+ expanded NK cells to infiltrate the BM.



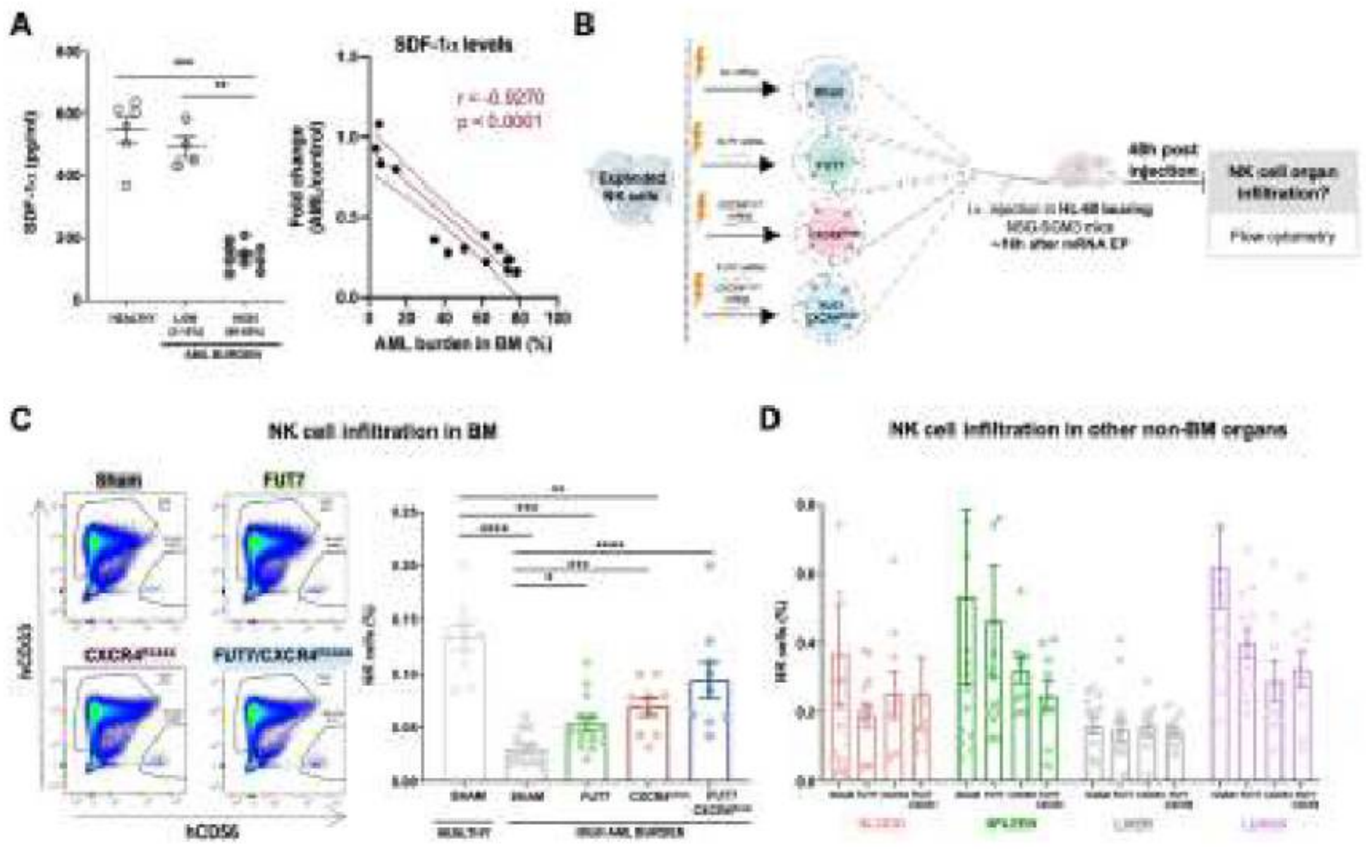
A Evaluation of changes in the BM endothelium (CD31 expression) during AML progression by IHC. Scale bar: 100 µm (upper panels), 50 µm (bottom panels). Legend: BM (bone marrow) and M (muscle). B Assessment of E-selectin expression (%) on the BM endothelial cells and quantification of total E-selectin+ BM endothelial cell number by FC. Bars, mean. Error bars, SEM. The Mann-Whitney U test comparing healthy (tumor-free), low and high tumor burden conditions was used for statistics. C Experimental layout for in vivo homing of adoptively infused expanded human NK cells electroporated with mRNA coding for FUT7 into (HL-60)-bearing NSG-SGM3 mice. Created with BioRender.com. D NK cell infiltration (%) in several organs 48 h after NK cell transfer, assessed by FC (n = 12/group). Bars, mean. Error bars, SEM. The Mann-Whitney U test comparing mRNA electroporated vs sham electroporated NK cells was used for statistics.

expression in the BM. The increased density and size of the vessels, as well as a more chaotic vessel organization, could be observed in patches associated with leukemia growth (Supplemental Fig. 3A). Given this data, we next addressed if FUT7-modified NK cells had improved BM homing capacity over control NK cells when infused into NSG-SGM3 mice with high AML BM burden (Fig. 5C). As shown in Fig. 5D, introduction of FUT7 resulted in significantly better BM infiltration compared to controls, while less frequently observed in blood, lungs and spleen. Hence, this data indicates that FUT7-mediated upregulation of sLe^x on the NK cells can help them better infiltrate leukemia-containing E-selectin high BM compartments and thereby partially overcome the poor infiltration observed for unmodified NK cells. The role for the E-selectin/fucosylation axis in our model was confirmed by infusion of unmodified NK cells from two donors with distinct baseline fucosylation levels into animals with natural gender differences in E-selectin upregulation following AML BM engraftment (Supplemental Fig. 4). Overall, we conclude that FUT7-modified NK cells significantly better home to the BM of AML-bearing mice due to the combination of increased fucosylation of the NK cells along with leukemia-induced E-selectin upregulation on BMECs.

Co-introduction of FUT7 and $CXCR4^{R334X}$ in expanded human NK cells nearly restores NK cell BM homing in AML-bearing mice

A reduction in SDF-1α expression has been shown to be one of the main causes for the impaired homing

Fig. 6: Introduction of FUT7 and CXCR4R334X in expanded human NK cells nearly restores NK cell BM homing in AML-bearing mice.



A SDF-1α BM levels during AML progression, assessed by ELISA and the correlation between tumor burden and SDF-1α BM levels. Bars, mean. Error bars, SEM. The Mann-Whitney U test comparing healthy (tumor-free), low and high tumor burden conditions was used for statistics. B Experimental layout for in vivo homing of adoptively infused expanded human NK cells electroporated with mRNAs coding for FUT7 and/or CXCR4334X into (HL-60)-bearing NSG-SGM3 mice. Created with BioRender.com. C NK cell infiltration in BM in tumor-free and tumor-bearing mice after mRNA electroporation, assessed by FC. Gating strategy for HL60 cells (live+hCD33+hCD45+) and NK cells (live+hCD56+hCD45+), and representative dot plots from one mouse in each group showing increasing NK cell infiltrations. D In vivo homing of ex vivo expanded human NK cells in several organs 48 h after cell transfer, assessed by FC (n = 9–15 mice/group). Bars, mean. Error bars, SEM. The Mann-Whitney U test comparing mRNA electroporated vs sham electroporated NK cells was used for statistics.

of cytotoxic lymphocytes to MM or ALL containing BM compartments [7,8]. Recently, SDF-1α has been also found significantly down-regulated in AML [24,25] Here we show, for the first time, that SDF-1α levels in the BM of NSG-SGM3 gradually drop with increasing AML growth (Fig. 6A). Given our recent insights that introduction of the GoF variant of CXCR4, CXCR4R334X, to NK cells results in that they also respond to low levels of the SDF-1α 19, we hypothesized that CXCR4R334X overexpression would help them better infiltrate AML-containing BM compartments with low SDF-1α levels. For this reason, we transiently introduced CXCR4^{R334X} alone or in combination with FUT7 per mRNA transfection to expanded NK cells and evaluated their ability to home to the BM and other tissues of HL-60 engrafted NSG-SGM3 mice (Fig. 6B). Due to the transient CXCR4^{R334X} upregulation following mRNA transfection [19], we performed sequential electroporations to match the optimal expression of both transgenes. As shown in Supplemental Fig. 5A, B, introduction of the mRNAs alone or in combination did not affect NK cell viability while quickly and significantly increasing the fucosylation level and/or CXCR4^{R334X} expression. Importantly, introduction of CXCR4R334X into FUT7-modified NK cells did not cause any alterations in the fucosylation kinetics while expressed to the same levels with the same efficacy. Moreover, NK cell cytotoxicity was unaffected in all conditions as assessed by co-cultures with both myeloid leukemia and EBV-LCL cell lines in the absence or presence of rituximab (Supplemental Fig. 5C, D). This was also the case for the expression of an array of cell surface molecules that remained unaffected (Supplemental Fig. 5E), corroborating previous reports showing mRNA electro-poration does not infer major significant changes to the NK cell phenotype [19,26]. When infused into NSG-SGM3 mice with high AML tumor burden (Fig. 6B), FUT7/ CXCR4^{R334X}-modified NK cells showed strong BM homing capacity compared to unmodified control NK cells (0.094% vs 0.030%) (Fig. 6C), approaching the potential of unmodified NK cells infused into healthy tumor-free mice (0.094% vs 0.14%). In contrast to NK cells modified with either FUT7 or CXCR4R334X that both had statistically significantly reduced infiltration capacities compared to unmodified NK cells infused into tumor-free mice, FUT7/CXCR4R33 4X-modified NK cells had a restored infiltration capacity of around 67% which was statistically insignificant from the control. Remarkably, the degree of NK cells was reciprocally reduced in several other non-BM organs, with a close inverse association between the infiltration level of NK cells in the BM versus the spleen (Fig. 6D). Interestingly, when infused under healthy conditions, the effect of these modifications was not as significant as in the high AML-bearing condition (Supplemental Fig. 6).

Discussion

Whether myeloid leukemia development negatively affects the BM homing ability of adoptively infused NK cells has remained unknown. Insights could critically impact on the formulation of future NK cell products to further sharpen their efficacy. We here for the first time demonstrate that AML growth directly impairs the BM infiltration capacity of infused NK cells. As AML progression was shown to gradually reduce BM SDF-1α levels, recapitulating what is observed in patients [24,25], while increasing E-selectin^{+} BMECs, we explored the potential of modifying ex vivo expanded human NK cells to express FUT7 for intrinsic forced fucosylation along with GoF CXCR4^{R334X} to better sense suppressed SDF-1α levels. These modifications redirected NK cell homing to the AML-containing BM via enhanced binding of upregulated E-selectin and distinct response to the low SDF-1α levels. Our data highlight the relevance of studying how AML-mediated perturbations impact BM homing of infused cell therapy products and how such perturbations can be utilized to more specifically redirect effector cells to the myeloid leukemia niche in vivo.

NK cell presence in the BM after adoptive infusion into patients with myeloid leukemia positively correlates with outcome [4]. Indirect data also show that AML and high-risk MDS patients receiving NK cells with higher CXCR4 expression have a higher likelihood of responding compared to those receiving an NK cell product with lower expression [3]. These data underscore the key role of proper NK cell homing to the site of myeloid leukemia to induce good clinical responses in settings of adoptive cell transfer. However, at the same time, studies have highlighted the relatively poor baseline BM homing potential of infused NK cells [27]. Our recent work has shown that NK cells manipulated to over- express CXCR4^{R334X} have enhanced BM homing capacity when infused into healthy tumor-free NSG mice compared to control NK cells [19]. We have also shown that infusion of ex vivo expanded human NK cells mRNA transfected to transiently express CXCR4^{R334X} significantly better target AML in the BM of NSG-SGM3 mice resulting in prolonged survival compared to mice treated with unmodified NK cells [28]. This mechanistically and more formally establishes a key role for proper NK cell homing to the BM via CXCR4 for treating myeloid leukemia. Nevertheless, these data were generated using a different and more aggressive tumor model than used in the current work. Due to the aggressiveness of the MOLM-14 cell line used in those mice, we started treatment three days after i.v. inoculation of the tumor cells in that model and although the infused MOLM-14 cells clearly established in the BM, the AML cells did not have enough time to induce perturbations to prevent NK cell BM homing. Given that untreated animals had about 20% AML cells in the BM when reaching pre-defined termination criteria, the role for myeloid leukemia-induced perturbations of the BM niche could simply not be studied in that model [28]. To better address the role of AML development in the present study, we here used the HL-60 cell line that similar to MOLM-14 establishes in the BM after i.v. inoculation before spreading to other organs but in contrast to MOLM-14 is less aggressive causing termination about 40-50 days from inoculation [17]. Importantly, in contrast to MOLM-14, HL-60 is relatively insensitive to NK cells, which is critical to our study as it avoids biased post-NK cell infusion analyses. However, this also implies that survival analyses comparing HL-60 engrafted mice treated with FUT7^{+}CXCR4^{R334X} NK cells to those treated with control NK cells cannot be adequately done. While the present study establishes proof-of-concept with focus on the mechanisms for improved homing late in disease when AML-derived microenvironmental changes occur in the BM, future studies will have to address the therapeutic potential of this approach and how it for instance contributes to reduce tumor burden and improve survival when adoptive NK cell infusion is combined with for instance an AML-targeting BiKE or TriKE, chemotherapy prior cell therapy or when the NK cells are equipped with a CAR.

AML cells can create a self-reinforcing leukemic niche to support their survival per se but also chemoresistance [9,10,11,24,29,30,31,32,33]. While E-selectin is constitutively expressed on the endothelial cells of BM and skin [21,22], stress conditions like cancer can induce E-selectin upregulation [9,10]. Moreover, this is associated with angiogenesis and increased microvessel density [30,34,35,36] that together with increased E-selectin expression is reported important for retention of leukemic stem cells in the BM [34]. In fact, the interaction between tumor cells and selectins has been proposed to positively correlate with disease development and is associated with metastasization [37]. Highly metastatic cancer cells often upregulate fucosyltransferases and present high levels of functional E-selectin ligands, leading to stronger interactions with E-selectin compared to the less metastatic subtypes. This has been reported for both solid tumors and hematological malignancies [38,39,40,41]. Further supporting the relevance of this axes for tumor development

and progression is the fact that the percentage of E-selectin ligand expressing primary tumor cells in both MM and AML cells have been documented to be higher in relapsed patients compared to newly diagnosed patients [42,43,44]. Hence, these data highlight how central the E-selectin pathway is but at the same time suggest that reprogramming effector cells to harness this pathway for better tumor targeting may be a viable approach in certain settings for some cancer. Whether this strategy is equally good or better than disrupting the leukemic cell adhesion in the BM by inhibiting AML cell binding to E-selectin and by that mobilizing them from their protective niches remains to be addressed [11, 45].

Based on the reasoning above and our data showing that ex vivo expanded NK cells have low basal fucosylation levels resulting in poor adhesion to endothelial cells due to weak E-selectin binding, we in this study explored how to increase fucosylation levels with the goal of redirecting NK cells to the AML. Previous work has used ex vivo fucosylation of HSCs, CTLs and CAR-T cells to increase their BM homing capacity [23,46,47]. Here, we introduce the FUT7 enzyme by mRNA transfection, which has been reported superior [48], so that the NK cells can themself generate functional ligands able to better bind E-selectin on the vasculature in the AML niche. As presented in this paper, the introduction of FUT7 generated highly fucosylated NK cells for over a week with robust capacity to attach to activated endothelium under shear stress. Although they did not have significantly better BM homing potential when infused into healthy tumor-free mice with relatively few E-selectin^{+} BMECs, their ability to home to BM compartments of AML-bearing mice was more distinct. Since E-selectin binding contribute to the initial tethering/rolling phase, while chemokine receptor ligation is needed for the second phase to activate the cells and prepare for extravasation following firm adhesion [18], addition of CXCR4^{R334X} is important to add. Hence, although FUT7 introduction had a strong impact on NK cell BM homing in the context of AML, it was not enough to restore BM homing to the level observed for unmodified NK cells infused into tumor-free mice. Instead, the combination with CXCR4^{R334X} resulted in more prominent extravasation and increased infiltration of adoptively infused NK cells to the AML in the BM. This highlights the need for proper chemokine receptor signaling in addition to a proficient initial tethering/rolling phase. Although our data indicate the integrins needed for firm adhesion prior to extravasation are expressed by the NK cells explored in the current work, future studies may have to explore if these can be adjusted to ensure full extravasation potential towards the AML niche of the BM.

Another aspect that could be considered to further enhance NK cell homing to the AML BM is to combine adoptive transfer of FUT7^{+} CXCR4^{R334X+} NK cells with chemotherapy that, beyond cytoreduce, also help restore SDF-1α levels in the BM while further upregulate E-selectin [8,22]. This could increase the potential of our approach further as over- expression of the CXCR4^{R334X} receptor has been documented to also work potently with normal SDF-1α levels [19]. Beyond this, one could also consider addressing the role of other chemokines and adhesion molecules such CCL3 and CXCL9/ 10 to promote infiltration into the proper anatomical location [29,49,50]. Indeed, the high upregulation of CXCR3 observed in our expanded NK cell product could be potentially relevant in this situation as well, since the levels of its ligands, CXCL9 /10, have been shown to be elevated in the BM of AML patients [51]. Deciphering the AML-associated microenvironmental cues in more detail in relation to NK cell homing would allow to design NK cell products with a stronger capacity to infiltrate AML-rich compartments [29,52]. This is not the least important for patients with chemorefractory disease but potentially also for patients with extramedullary disease that represents a unique challenge [53]. Depleting or blocking certain chemokine receptors could also be beneficial to avoid competition of NK cell infiltration into other non-AML engaged organs such as lungs, liver and spleen or their mobilization from the BM [20]. In relation to this, S1PR5 depletion could help in retaining NK cells within the BM, since the S1P concentration is elevated in the plasma of cancer patients [54] and is higher compared to the concentration in BM of AML patients [55].

That we cannot address tumor reduction and subsequent survival in the present model represents a potential limitation of our study. Moreover, mouse models per se comes with limitations. Despite relatively clear data showing the potential of capitalizing on CXCR4^{R334X} and introduction of FUT7, infusion of human cells into mice may inherently miss key data as the cross-reactivity of certain receptor ligand systems may not be complete. Nevertheless, the current model may serve as good grounds for assessing key insights. Future clinical exploration may better establish the therapeutic potential of the presented approach. Such studies may also shed light on data from individual patients with heterogenous disease rather than from genetically identical bred mice inoculated

with an immortalized myeloid leukemia-derived tumor cell line.

In summary, our study presents the first evidence showing that AML development negatively impacts the ability of adoptively infused NK cell to infiltrate the BM. The data also reveal that genetic modification of NK cells to express functional E-selectin ligands and the GoF CXCR4^{R334X} can rewire their homing to the AML in vivo. Strategies to identify additional tumor-associated microenvironmental changes and how to capitalize on these to redirect adoptively infused cell products to the site of disease may be a promising approach to further improve NK cell-based immunotherapies of cancer. Future studies are warranted to address translational potential of this concept.

Data availability

For original data, please contact mattias.carlsten@ki.se.

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M.C. conceptualized, coordinated and financed the study; M.C. and L.S.O. designed the study, interpreted the data and wrote the manuscript; L.S.O. performed the experiments with support from A.A. All authors reviewed the drafts, interpreted data and approved the final manuscript.

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continued from Nov./Dec. 2023 edition

The features of the Pharmaceutical Industry of the future in Nigeria - The Drugfield Perspective

In continuation to last year Nov./Dec. 2023 edition piece interview with Pharm. Olusola Akande, some part of the interview applied to situations of 2023 and however may not be held strictly to the 2024 situation for instance the foreign exchange realities of 2023 comparable to the prevailing forex situations. Other areas may point to the current energy input cost (diesel, petrol etc.) as a major contributor to the difference between then 2023 and now. I hope you'd enjoy the rest of the interview.

Pharm. Olusola Akande

That could also make you a bit more competitive against the Indians and the Chinese. Okay. Let's look a little bit away from the industry. Let's look at your responsibility as a marketing director. Yes. In those areas, what do you see as challenging in your responsibility? Especially as a Marketing Director?

Now, the greatest challenge in marketing is the marketing cost. Marketing is an expensive venture. It's not cheap. For example, if you have over-the-counter products and you want to promote them, you may decide to use above-the-line strategies like Billboards, BRT exposures, TV, radio, social media, etc. By the time you go into that, you will realize that these things are not cheap. Sometimes you sit down and watch TV adverts, to you, an interesting advert has just been watched but to the person who paid, it could be an expensive venture. And one thing about marketing is sometimes consumer behaviors cannot be predicted, so the money you are spending is not guaranteed to return especially if your plans and executions are faulty. So, there's an element of risk involved. You need to do a lot of brainstorming before you embark on any marketing project.

The cost is a big challenge for most companies, especially pharmaceutical manufacturing companies where you always have limited resources being competed for by many other issues like Forex, procurement of API, machines and equipment, etc.

In that area, we've spoken of promotions in the marketing sphere. And there is distribution. Before we go to distribution, there is a new media. The one we call new media is social media. Everybody knows that has democratized almost everything in the media. So, one can see what one wants to see. And sometimes, the ease of seeing it is much more enhanced. I can see it in my bedroom. I can see it in my toilet. Not waiting to see it on the conventional TV screen, or come to a special place to see it on the TV. Are you not exploring that area? Is it not competitive with the mainstream media?

Yes, we are. Well, let me say it's more cost-effective. It's not free. But it's more cost-effective when it comes to stuff like that. But when you want to do an encompassing promotion, you need to explore all the avenues, including social media or new media and traditional media. That you are doing new media should not stop you from using traditional media, because you want to reach everybody, especially if it is an OTC product. For example, last year, we ran a campaign for one of our products - Neurogesic ointment. It's an ointment for managing pain. We did BRT promotions, TV advertising, radio promotions, and social media campaigns. You need to do everything. An Okada man most likely would not get your advert on Facebook or Instagram, but they can see it on BRT. So, it depends on the product and who you are targeting.

Okay, let's look at distribution. Since we're talking about marketing and distribution, obviously it's your responsibility to push these

products to them. I don't know your process though. There is the land which comprises the road, and the rail, and there is the air. Let's leave out the sea. In those areas, where do you have the most challenging distribution channel? How challenging is it and which process did you use in surmounting the challenge?

Initially, we were distributing our products ourselves. We have trucks. All our products were being exclusively distributed by our trucks. We have a whole unit that is in charge of that. But because of the challenges associated with this like truck maintenance, managing the drivers, etc., we decided that we needed to outsource the distribution process to external logistic companies. We engaged them to start taking these goods to our various depots. All you need do is give them the address, and they will take the products there. We have an agreement with three or four logistics companies. They go as far as the Northeast, Northwest, Southeast, and South-south parts of the country to deliver our goods. This process has impacted our efficiency. So that is that for the road.

Another challenging one is when you want to export, you know we do export. So, for example, when you want to export through the sea, your product could be at the port here in Nigeria for weeks. All manner of stories may come up: The ship has done this, there is no space, and there is no this and that. So sometimes, when we don't have any other choice, we explore the air. But the air could be expensive too. If they ask you to supply at a particular price and you base your calculations on the sea freight, and at the port, you have a challenge and you want to switch to air. By the time you input your cost, you realize that the price you quoted may not be profitable. You can't tell them, it's N20 today and tomorrow you change to N50. You may end up losing money not budgeted. So, these are some of the challenges we face.

All right, is there any additional thing you want to say about the marketing area? Is there anything we're leaving out that you want to speak of?

You know, when it comes to marketing you must always remember you operate in a highly competitive environment. The Pharmaceutical industry is highly competitive now, especially with the imported brands. If you have a locally manufactured Antimalarial for example, you have other locally manufactured brands to compete with, and also imported brands. So constant innovation to keep abreast of the current market situation is very necessary. You need to be creative in your marketing strategies and always know who you are targeting for a particular product and then deploy the necessary marketing tools to achieve that. You know, we have two types of products, over-the-counter and ethical products or what people refer to as POM (prescription-only medicine). With the POM, you reach the end-users through a third party which most of the time are the professionals; Doctors, Nurses, Pharmacists, etc. So you reach the end-user through those people. In that case, there's a different marketing strategy for that. But if it's an OTC, you may not need professional recommendations or prescriptions to reach them just like our mosquito repellent gel, - Reptogel. The way you market those is different. You need to be innovative. You need to streamline your marketing process and tailor it to what your needs are so that at the end of the day you hit the right target.

Before we go further, let's look at business clime in 2023. We are well into the fourth quarter. What are your expectations of the Nigerian pharmaceutical industry before the year 2023 runs out? Are there targets the industry set as a group that you desperately will need to see achieved before the year runs out? As it relates to you as Drugfield?

Yeah, of course. Organizations at the beginning of the year always set targets and goals that they intend to achieve for that year. If you look at 2023, as a country, we had a general election. We had a transition from one government to another. When the new government came, they introduced certain policies that disrupted so many things. For example, the removal of fuel subsidies, floating of Naira and having a single Forex rate. These disrupted many plans most companies had at the beginning of the year. Then, as an industry, we met with NAFDAC recently and there are some goals we set before NAFDAC. The ease of getting our Dossier approved is the number one goal of most manufacturers now because this will determine how many products you can register. They have a window that is opened every quarter and there are limited numbers of dossiers each company can submit per quarter

Is the industry trying to defend that collectively, or is it an individual cry?

Most of the time, it's an individual cry but once in a while, when we have an opportunity to interface with the regulator, we always mention it and it becomes a collective issue. The PMGMAN has a management team led by the Chairman and we also have an executive secretary. So, we interface with NAFDAC from time to time and this has helped.

All right. Let's look at the economic policies. From what you've said so far, the economic policies have roughened the waters. Do you agree that this present economic policy is capable of moving the pharmaceutical industry in Nigeria forward? Do you agree, do you believe or do you think it can move the industry forward from where it is? Maybe given the kind of targets you have, and maybe given the projections the pharmaceutical industry–(as an umbrella word) has.

Well, if you look at some of these economic policies, they have their pros and cons. And I've listened to different arguments, both for and against. So, it depends on which side of the divide you are. (interjecting)-Of course, you will be on the side of favour for your industry. Yes, but if you look at some of these policies objectively, before this administration came on board, the last figure we heard that was spent on subsidy was about seven trillion, - fuel subsidy. Whether it is real or not, that's the money purportedly taken out of the system for that purpose. Which sane country will continue with such? None. How can you spend seven trillion Naira on subsidies? And the seven trillion means the money you are spending on fuel subsidy is more than your budget for health, more than your budget for education, more than the defense budget, more than any budget. No budget has seven trillion. I mean, it's senseless. If you look at the election time, all the major contenders unanimously agreed that the subsidy has to be removed. However, the removal of subsidies will come with a lot of consequences. That is where the pharmaceutical industry comes in. The purchasing power of people is being eroded every day. These are the people we expect to buy our products. We have found ourselves in a situation where people go to hospitals only when they are seriously sick. Stuff like medical checkups to prevent being sick or early detection of diseases are taking the back seat because most people can't afford them. Medical checkup? I have not eaten and you're telling me a medical checkup. (Reaching out for some products). We have some products that are cosmetic in nature. We have an aftershave. Neo-Presol is an aftershave lotion. With a product like this, if you have not eaten, will you want to do aftershave? Some of these economic policies have affected the purchasing power of the average citizen. Nobody is saying you should not take away the subsidy, what palliative measures are you putting in place to alleviate the suffering of the masses? Fine, the subsidy has gone. But what are you bringing on board? Are you increasing people's salaries beginning with minimum wage, as a reaction to the fuel subsidy removal? These are stuff they have to sort out. Then, the second major policy, at least so far in this government, is the issue of Forex. We all know that having a dual exchange rate window breeds corruption. People were getting a dollar at about N415 official rate from CBN and selling at above N700 on the black market. And if you're highly connected, they could give you up to $2 million, or even $3 million. That was why they were saying the former CBN governor was just churning out billionaires who have no offices. So, nobody wants such to continue. Now the way they have floated the Naira, letting the forces of demand and supply determine the price is good but affecting the manufacturing industry. For example, some forward letters of credit were opened when the dollar was around 415 official rates with the hope of paying the suppliers in 90 or 180 days. The suppliers had released the goods only for the naira to be devalued before then. So, as a manufacturer, you had received the API, used it to manufacture your products, and based all your calculations on 415, only for you to pay the supplier at a higher rate. In that case, you need more naira to pay off the supplier.

So, in any case, it was like an emergency. It wasn't expected. It's about two months now from the time the shock came; you would have recovered from it so far. Because of the way you're talking, some of those shocks have been absorbed by the publication of what the corporate world lost in the process. Well, I'll see it as a shock that has been absorbed. Is there any process you're putting in place to either recover or to get back on track?

Yeah. The only way to recover and get back to the line is to adjust your price, which is also not too good because the purchasing power of people is being eroded daily. Invariably, the consumers would have to pay for it. You can't sell below your production cost. Almost every company has adjusted prices. That's the easiest way to react to that.

Let's look at the now almost popular trend in Nigeria, the one we call JAPA syndrome. In the earlier days, we experienced a massive brain drain, especially at the medical level; Doctors, Pharmacists, Nurses, and other medical practitioners especially experienced the worst exodus. However, some non-professionals are also migrating. But now it's expanding, it's cutting across the board. Is it affecting the pharmaceutical industry? And how has it affected it? Contrasting it

with an average fresh graduate who wants to go abroad, are the existing pharmacists moving in droves the same way as the doctors?

I don't think there is any industry that is not affected by that JAPA syndrome. On a lighter side, I was saying jokingly with one of my friends that JAPA had been in existence for so long. JAPA is in the Bible. Remember how Abraham went to Egypt, how Isaac went to Israel. Even God instituted JAPA when he said to Abraham; leave your father's house and JAPA (general laughter). It's an old practice. But it took a dangerous dimension towards the end of Buhari's regime because people are now doing it for economic survival. In the past, people would travel simply for the experience of it. I'm a doctor in Lagos, let me go and practice in New York and experience other climes. But when you want to travel abroad just for mere survival, that is where the issue is. And people are now doing that in droves.

The BREXIT matter that happened in England is not helping matter because when the UK exited the European Union, many of the citizens left. So, they needed people to fill in the gap and they saw Nigerians, Indians, Chinese, and other Africans as cheaper alternatives. At the time they were recruiting nurses in Nigeria. They used to set up desks in designated centers in Nigeria to recruit them. That was so daring. Even Saudi Arabia came, and Nigeria didn't do much. Then COVID-19 affected Canada a lot. So, they also realized that they needed a lot of foreigners. They have a very large land mass in Canada but few people to fill in some of these areas. They also need a lot of people in their country. You realize that 70% of those who JAPA either went to the UK or Canada.

So, this affected so many industries. There was a guy in one of the banks who told me that many of his colleagues at work have traveled. In my place here, (pointing to the window opposite him), one of my managers, just last week, has JAPA. Our export manager and one of our brand managers, have also JAPA. There is no asking the question of how it is affecting you. And these are people you have trained for 10yrs, 15yrs, and more. The last guy that left spent about 15 years with us. Each time we had clinical meetings in the hospitals, the brand manager was the one who would go to the hospital to do the presentation for the medical professionals. He has gathered the experience over the years. He knows the right answers to every question. Where do you want to get such a replacement? Even if you employ somebody now, you have to start from A for Apple, and B for Ball. It is a big challenge.

It will affect other pharmaceutical companies in the same way. So, are you doing anything to forestall that? Like giving more incentives in your capacity.

In our capacity, I don't think there is much we can do to stop that because the reasons why people are doing that are far beyond what we can offer solutions for. It is largely for economic reasons. Some will tell you lack of infrastructure, lack of this and that. When people are pushed to the wall. – (interjecting) -Infrastructure comes even tertiary. The primary thing is pay pocket. Yes, they are all interwoven. You can say I don't like the road, I don't have water, and electricity is not stable. But if I have enough money, I can make myself comfortable; I can get myself some electricity. But let me tell you how they are related. In Drugfield, on power alone, we spend several millions of naira every month. If the government is giving us regular power, and we are just paying less every month, the balance we could use to increase our staff salary. Those who have more money in their pocket would think less of JAPA.

That is the area I am going to. What you are doing to stop it?

If you increase somebody's salary, and the next month they devalue Naira again, and prices of things go up again, you are back to square one. What are people looking for? Nigerians are not asking for much. Let them be able to afford basic things. Let a young graduate be able to drive a car of his own immediately after he starts working. In other climes, for some of my mates, once you start working, you go and pick any car of your choice as a pharmacist. The same thing goes for a house. You don't need to own 100% of the money to buy a house. But here, you want a young graduate who has just started working, whose salary is N200,000-Naira, to face every economic upheaval successfully, you know that can be very frustrating. I know how much I pay for my children's fees for secondary school. In Canada it's free, In the UK it's free, -Free! Those are the things people are running away from. Except if you don't want your children to become something better than average, to put them in a public school here in Nigeria is not the best. No matter how bad it is, try and put them in private schools, though they are very expensive. I don't think primary and secondary education should be that expensive. That is the economic state and reality of our country now.

All right, let's advance from JAPA. All around now, Alternative medicines are gaining ground. Much more ground than they used to,

comparable to the pharmaceutical Drugs. In those days it used to be Herbalists. If it is not drugs, then it would be the herbalist and his Herbal concoction. These days there are alternatives. There is the advent of Supplements; both food supplements and some other kinds of supplements, and the trend is almost exploding. They are all struggling for market shares with pharmaceutical drugs. Is it affecting you negatively?

You know, when they say Alternative medicine, it means there was something in place before, and they now went to look for the alternative. That is why they call them Alternative medicine. And you don't look for alternatives if you are getting the best result with the one you are using. Yes, Alternative medicine is gaining ground, and what most pharmaceutical companies are doing now is to join them in marketing Alternative medicine. – (interjecting: -That means if you can't beat them, join them). So, they call them Nutraceuticals. -Some of those herbals and the rest, they call them all Nutraceuticals. – (interjecting:-I have also heard Herbaceuticals). So, you now have a situation whereby a company is subdivided into two, one is the pharmaceutical division; the other would be the Nutraceuticals division.

Okay, you've answered the second prong of the question of; what measures are you taking. You've also confirmed that you are also joining the alternative medicine race. Now, there is a general belief, -maybe not general. But the understanding is that the Nutraceuticals - (like the Herbal and nutritional Supplements) - help enhance full recovery from the ailment processes, through the restoration or reversal of health conditions. While drugs have the one duty of management of health conditions, but not reversal. Is it true? Since you are fully into it now, I believe you should know enough about the effects and benefits of Nutraceuticals.

Many of these Nutraceuticals make use of natural plants, while most pharmaceuticals use synthetic Chemical compounds. In the recent past, campaigns started that these chemical compounds have side effects, they have consequences, so why don't you go natural? That's how the shift towards the use of Nutraceuticals started. But the problem or the limiting factor with most of these Nutraceuticals is that there have not been enough research and studies to back up some of these claims. If you take an anti-hypertensive drug, maybe Amlodipine, for example, and you open your internet, you'll see a lot of work that has been done on Amlodipine both locally and abroad. Clinical trials were carried out and the likely side effects on Whites, blacks, or Caucasians are all available. So, a lot of work has been done in this regard. Unlike the Nutraceuticals where you have limited research. I'm not saying there is no research. Some research you have them carried out on whites. Some are not carried out on Africans. And sometimes race or colour affects the effects of some of these drugs. As I earlier said, especially in this part of the world, the belief system also matters in some of these things. Some people would say if I take it, everything will just go, while some people will take it and have no effect. So, there is not enough data to make any conclusion on some of these Nutraceuticals.

If the old days, Professor Dora Akunyili –of blessed memory- is still alive, probably, there would probably have been more push for Nutraceuticals. Because one of those times in her days, I watched her speak on drugs as against herbs. She was explicit about it; you know she was a pharmacologist. While she didn't say to leave drugs and focus on herbs, she tried outlining the advantages of the two: - Organic herbs against pharmaceutical drugs. She said: that while that herb will blend with your system because it's organic like the human body is, the other one is like a foreign body sent into your system. And after some time, it will just take up residence in the body. So, it's not actually like it's a lie that there are side effects.

Yes, there are side effects. But the major problem with some of these Herbs initially was the issue of standardization. Even when we were growing up, if we had malaria, they'd tell us to just squeeze DOGO-YARO-(Neem) leaves and drink the water, just take one cup. But the issue is, what quantity of it do I need to drink to get the necessary effect? There has not been extensive research on many of them. -(Interjecting)-The Indians are high on that; they are researching them considerably. Yes, they are the biggest player when it comes to Nutraceuticals, I can tell you. They are standardizing it. Some of those herbal stuffs are the ones being standardized. -(Interjecting)-They call it Ayurvedic practice in India. Yes, Ayurvedic. China is also doing good work in that regard too...

Okay, so it's not really hard on the pharmaceutical industry in Nigeria because you are already playing along. That's the long and short of it, isn't it?

But you know it's easier to register Nutraceuticals in Nigeria. You don't need a Dossier; you don't need a Drug Master File. -(Interjecting)-why? ...The manufacturing process is not as cumbersome as the

pharmaceuticals. They are not chemical compounds that you need to do the profile pathway and all that comes along with it. There is something called a drug master file. If you want to register a product, you'll be required to bring a drug master file of the API. The manufacturer of your API will supply the drug master file to NAFDAC. They call it DMF, - Drug master file. It's going to show the pathway of how the API is synthesized, to be sure that the product is genuine knowing that the easiest way to make a fake product is for the API to be fake. Once the API is fake, the product is already fake. NAFDAC wants to ensure that the API is not fake, so they'll ask for the DMF - Drug master file. But with Nutraceuticals, you don't need all those documents, that's why their registration is easier.

A few more questions here, we'll be looking at the road. Okay Let's look at the upcoming ones in school, where their eyes are set, what they are targeting, the scarcity and availability of various arms of pharmacy, we've spoken about all those. If you are a representative of probably the pharmaceutical M.A.N., or the entire pharmaceutical industry in Nigeria, and you have to advise them, what will you send out as a message to them?

Number one is focus. There are different aspects of the pharmacy profession, you have the hospital pharmacy, the industrial pharmacy, the community pharmacy, and the academic pharmacy, and you have various arms, like six or seven different arms of pharmacy wherein one could practice. Know where you belong, know what you want. Not that you do hospital pharmacy for three years, and say you are tired, you now go to community pharmacy for four years and say you are tired, you now say it's industrial pharmacy you want to work now. Because I have friends that do that. So, you must know what you want from the onset before you leave school. You can only excel where and in what you have flair for, some people like the marketing just like the way I'm doing now, and some people like taking care of patients, maybe community pharmacy is in their nature. Know where you belong, go for it, and stick to it. Stick to it until you become a master in that area. I've always liked marketing, and since I graduated from school, I've always done sales and marketing. So, for a young person, be focused, and know what you want.

Let's tone it down a little more now. You've spoken about your children and how much you are putting into them. Are you influencing any of them to take up your kind of profession? Are you hoping to? Even if they're - Probably- not up to the age where you would begin to influence them? Or maybe they've gotten to the stage where you could well influence them.

One of the things I like to imbibe in my children is for them to be independent. –(Interjecting)- Okay, that's good in their thinking. Yes, to be independent-minded in their thinking, and just get guided. So I'm not an advocate of you must go and study this course. And I do not prevail on any of them to say you must follow my footsteps, No I don't. My first daughter says she wants to study medicine in America, and I say okay God will help us. She said she wants to be an "ANESTHESIOLOGIST". You see, God created us differently, and He created every one of us for a purpose. So, I don't want to impose my purpose on their own. We are just caretakers; God is the original owner of our children.

Okay let's look at your corporate social responsibilities as a company, if there is any. But I don't believe there are, right?

As a company, yes of course we do a lot. -(Interjecting)- Are you willing to share some of them? Yeah! We have a foundation that is dedicated to that. They have staff, they have people running it. It's being funded by the company – Drugfield; we call it "AJOKE FOUNDATION".

The AJOKE FOUNDATION, do they have limitations? As in, do they have areas they don't cover? Or anything they won't get involved in?

Yeah, we give scholarships to University, Polytechnic and Secondary school students - (interjecting)-That's good to hear. Widows who don't have means of paying some bills. It's a whole department on its own. - (Interjecting) - But it's not, it's not widely known... Why the lack of publicity? Well, we make little publicity about this

No, I disbelieve that, I beg to differ rather. Because that alone could tie to the attraction to your drugs, or rather to your products. And it could also inform a particular liking to your line of products if you do the required publicity. Okay, let's say I'm speaking from the mind of the public; If these guys are doing this much, then let's patronize them because they are giving back. Some people may have that kind of mindset you know. That's why I'm looking at tying it together

Yeah, I understand what you are saying. There are benefits inherent in advertising. But another way of looking at it is that you have a set of Indigent students that you want to help and to help them you go and bring cameras and Television to come and show them to the world that these are the indigent students

I want to help. It may sound somehow to some people. God forbids! Assuming I'm an indigent student and somebody wants to give me a scholarship, and he's going to carry the whole world to come and see, I may not like it. But that does not mean we don't publicize, I mean, in our little way, we advertise our CSR.

But you can help that situation; you can help that situation by combining the indigent students with the non-indigent except you say it is only for the indigent students. Is your CSR only for indigents?

Well, there is a way they screen, though I don't have all the guidelines I know they screen. Preferences are always given to indigent brilliant students, and Orphans; - (interjecting)- that's when it comes to scholarships right? Scholarships, yes, if somebody is sick you don't need any criteria, we just intervene. So, we do that a lot, we donate drugs even to government agencies -(interjecting)- They should be publicized. I believe they should be publicized. Yeah, we do some of these, for example, during World Glaucoma Day a lot of hospitals ran free glaucoma screening for patients, and we supported them with drugs. The last one we did, we partnered with the Lagos State Government, and we gave them some drugs too. Lagos State Government made some noise around these themselves. Many of these are on our social media handles.

Okay! There's an area I left for the last. On your website, amongst some other features-, I just singled out the NAFDAC Excellence Award that is showcased there among other recognitions. How would you assess the recognitions or the awards that have been given to you in terms of encouraging or probably propelling you to work harder, or to give more?

Some of these Awards like the NAFDAC Recognition and all the like; are just reminders of the fact that at any point in time, we must stick to the quality of our products. Our slogan is quality products from a very sure source. - (Interjecting)- That's very good. We know we got some of these awards because we are sticklers for quality, and the awards always remind us that we must not depart from that, we must always adhere to the quality of our products. So those are the things surrounding our recognitions and awards.

Okay, you are a member of the Pharmaceutical MAN –otherwise known as PMGMAN. If you happen to be the leader of PMG-MAN or of the Pharmaceutical Society of Nigeria - I'm looking at the Apex body, which of them is the Apex body?

The PSN is the umbrella body because it comprises manufacturers, community Pharmacists, and industrial Pharmacists. Everybody is under the umbrella of the PSN.

If you happen to be the head - which is not impossible, what would be your target for change, what would you want done differently? From what you have experienced so far from the leadership and the industry, what would you want done differently? What would you push for?

There are so many things to push for, so it depends on the sector you are talking about. -(Interjecting)- I'm looking at the industry as an umbrella because with that umbrella you are responsible for all the sectors. Okay, for example, one of the things I would advocate for, which we've been collectively campaigning against, is the issue of drug distribution. There is an awkward drug distribution system in Nigeria. Look at the open market, drugs should not be sold in the open market. -(Interjecting)- Is it different from the Prohibition list you were pushing for? No, the prohibition list is the products that Local manufacturers can produce that should not be allowed to be imported. I'm talking about local distribution. Look at Idumota -I don't know if you have been there before-, drugs should not be sold in such an environment. Look at Onitsha – headbridge, the same thing with Kano. There were attempts to stop drugs from being sold in the open market, and that those places should be relocated to a more decent and conducive environment. It's only Kano that has attempted a change, they built a mall and asked that everybody should relocate to the place and they also gave a deadline, I was reading it. But Lagos and Onitsha, have refused to do that, and 60 to 65 percent of the drug market is controlled by those two places. So, If I have my way as a PSN chairman or head, that's one of the things I would push for. Most of the guys selling drugs in those markets are not even pharmacists. Most of them didn't even go to school, they just saw drugs trade commodities like clothes, shoes, land, etc. It should not be so, -(interjecting)-Okay, That's the major thing you will push for? That's the major thing I would push for. Then when you talk of hospital pharmacy, how do you ensure that our colleagues in that area are not relegated when it comes to decision-making within the system, -remunerations, cadre, and all that stuff, which is a major challenge? When I was doing my internship at UCH the fight had been on before then, how can you give doctors an X-amount of salary and give pharmacists a Y-amount? The disparity is too wide, even up

till now, so those are issues we have to fight for. Until recently a pharmacist could not become a consultant. The question is why not? -(Interjecting)-and what was the reason then? Well, you know the doctors will always fight back in a kind of battle of supremacy. So, when it comes to the industry, we talk of some of these issues I have mentioned - reduction in tariff, access to forex, Prohibition list, and so on. As a PSN chairman, these are the things I would push for.

That's good, it's what you people are fighting already. We are going home now. What are your likes, Let's go!

Football. I love watching football. I grew up in Ibadan, so in my growing-up years, my dad was a diehard fan of the then-IICC. -(Interjecting)-Yes, I remember IICC. They're now 3SC –Yes. IICC of those days. So right from my primary school days, my Dad used to take me to the stadium every Saturday, to go and watch football matches. So, I built my interest in football through that process. I have always been a fan of football right from my childhood up till now. I have travelled to Manchester a couple of times to watch matches. I support Manchester City so I used to go.

Are there dislikes? Within your personal preferences? Are there dislikes too?

Yes, in terms of personality, I dislike people who are not honest, people who are not straightforward. It's very difficult to cope with them. Unfortunately, you have a lot of them around and they are all disguised. But I've developed a means of managing them. It pisses me off when you're dishonest. Well, I guess I'm not the only one who feels that. But sometimes I remember that we are in a world that has a mixture of persons, a collection of different minds, and different understandings.

What's your favorite food?

Rice and plantain. When it comes to food, I'm a very rare being. I don't eat what most people eat -(interjecting) - Rice and plantain are usual. Yes, but I've never eaten swallow in my life -(interjecting)-W.H.A.T! That's why I said I'm a very rare being. Okay! Hear this, I've never eaten meat in my life - I've never eaten chicken, turkey, Snail, etc.

Then what do you eat? Or have you been putting some wire and charging up your protein with electricity? (General laughter) What's your source of protein?

I eat only fish. -(Interjecting)-Wow!!!, Fish only? Yes, exclusively fish. Only fish. Maybe recently I've started eating shrimp and prawns

You are a rare being indeed. What informed it? Is there any reason for that?

Yes. My mum told me that each time she gave me some of those things I would vomit. If she gave me Amala, I would vomit. I've never eaten pounded yam and all that stuff before. It's not that I stopped; I don't know how they taste. -(Interjecting)-And she allowed you to follow that path? She used to say leave him alone, when he grew up, he would change and start eating them. She probably thought she was helping me. I now grew up, and I don't know how to eat them.

What's your most attractive colour?

Sky blue. -(Interjecting)-Is there a reason for that? The skies are blue Do you like brightness? When I was growing up, I used to have a shirt, a sky-blue shirt, I loved that shirt. When I was in Primary school, I was turning ten years old. So, my dad told me that if I came first in the class, he was going to take me to London for vacation. I think I was in primary four then. So, I tried, I came first, and then he took me to London. I celebrated my 10th year Birthday in London. My dad was the General Manager of the Housing Corporation in Oyo state, just like the LSDPC of Lagos state. He bought me a blue shirt, in London, he also bought me a brown suit. I used that shirt for my birthday. Since that time, I fell in love with that color, -sky blue. -(Interjecting) - So that's what informed your love for sky blue? Yes, that's it. So that's how I came to love sky blue, and it became my favorite color. I love sky blue.

IN CONCLUSION:

Alright!!! Pharmacist OLUSOLA.... It's been nice speaking with you. (Pharm. Olusola-. Thank you very much). If I were to speak on this strength, I would say let's do it again. -(Big laughter from both sides)- But it's not always going to be like this all the time. It's been a nice day well spent with you; you've touched on various areas. I hope we're able to explore the areas that are relevant to you and even beyond. Are there areas you didn't touch? Because I made sure we captured every area that speaks of the company -Drugfield, Yourself, and the Pharmaceutical industry as a whole.

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Sociodemographic profiles and organ damage accural in the Black Women's Experience Living with Lupus study

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Abstract

Objective

Black/African American women with systemic lupus erythematosus (SLE) experience greater organ damage and at younger ages than white women. The objective of this study was to advance research on SLE inequities by identifying sociodemographic risk profiles associated with organ damage accrual specifically among Black/ African American women.

Methods

Latent profile analysis was conducted among 438 Black/African American women with SLE living in Atlanta, GA and enrolled in the Black Women's Experiences Living with Lupus (BeWELL) Study (May 2015 to April 2017). Proportional hazard and Poisson regression models examined prospective associations between sociodemographic profiles and the timing and degree of organ damage accrual over 2 years.

Results

Four profiles emerged: (1) "Younger/ Lower SES with Uncontrolled SLE" (44.8%), (2) "Older/Lower SES with Uncontrolled SLE" (23.3%), (3) "Mid-SES with Controlled SLE" (19.6%), and (4) "Higher SES with Controlled SLE" (11.2%). Approximately 42% of participants experienced new organ damage during the follow-up period. Proportional hazard models indicated that "Older/ Lower SES with Uncontrolled SLE" participants were at greatest risk of new organ damage (HR = 2.41; 95% CI = 1.39, 4.19), followed by "Younger/ Lower SES with Uncontrolled SLE" participants (HR = 1.56; 95% CI = 0.92, 2.67), compared to those in the "Higher SES with Controlled SLE" profile. Poisson regression models revealed that these two groups also exhibited greater organ damage accrual (b = 0.98, SE = 0.24, 95% CI = 0.52, 1.44 and b = 0.72, SE = 0.23, 95% CI = 0.27, 1.17, respectively).

Conclusions

Black/African American women with fewer socioeconomic resources and uncontrolled SLE are at greatest risk for increasing disease severity over time. Social inequities likely contribute to racial inequities in SLE progression.

Introduction

Black/African Americans in the U.S. have higher prevalence of systemic lupus erythematosus (SLE), experience faster disease progression, greater disease severity, and worse SLE outcomes, including increased mortality that also occurs at earlier ages, compared to their white counterparts.[1,2] Although genetic risk factors for SLE exist, their role in explaining racial inequities in SLE is limited.[3,4]

SLE activity is driven by inflammatory mechanisms and, when uncontrolled, results in irreversible organ damage, such as kidney failure, cardiovascular disease, and pulmonary disorders.[2,3,5] Organ damage accrual and the rate at which it occurs are important SLE outcomes that predict disability, reduced quality of life, and

mortality.[3,6,7] For example, organ damage accrual within a year of diagnosis has been linked to a 3–4 fold increased risk of mortality, and existing damage increases risk of new damage accrual.[8,9] Several studies have reported that Black/ African Americans with SLE are at greater risk for organ damage accrual and mortality.[10,11] Black/African Americans also experience higher rates of initial damage (i.e., transition from none to at least some) and damage accumulation (i.e., transition from some damage to greater damage), compared to white SLE patients.[12]

Emerging research suggests the causes of racial inequities in SLE progression and outcomes are rooted in social inequities rather than biological differences in disease susceptibility.[1,4,13] For example, SLE disease activity is sensitive to psychosocial stressors unique to the lived experience of Black/ African Americans, such as racial discrimination and other forms of racism-related stress.[14–17] Other research has found that Black/ African American women with SLE living in racially segregated neighborhoods face increased risk of depression—a common comorbidity associated with organ damage in this population.[18,19] Structural racism also undermines socioeconomic mobility for Black/ African Americans, which in turn reduces access to health promoting resources and increases exposure to risk factors for SLE disease progression, including organ damage accrual.[20–23]

Most research on correlates of organ damage accrual has focused on clinical (e.g., disease activity, medication use) or basic demographic (e.g., age, gender, race/ethnicity) factors. A limited number of studies have identified poverty and socioeconomic disadvantage as risk factors for damage accrual.[10,20,24] However, much of this work has been conducted in predominantly white or racially diverse samples with low proportions of Black/ African American women. These limitations mask within-racial group variability and preclude the identification of sociodemographic risk factors specifically among Black/ African American women, a population that experiences disproportionate SLE burden. Moreover, most studies have relied on convenience or otherwise self-selected samples that may have different sociodemographic characteristics from the underlying population of interest. When attempting to identify subgroup patterns within a population, person-centered approaches (i.e., latent profile analysis) offer advantages over traditional variable-centered procedures (i.e., regression) by considering the intersection between multiple disease and sociodemographic characteristics and how they may cluster together. Profiles that emerge from a latent profile analysis (LPA) may be useful in clinical settings to identify patients at greatest risk of organ damage.

To our knowledge, there are no prospective studies which identify high-risk sociodemographic profiles of organ damage accural specifically among Black/African American women, despite this group being at greatest risk for accelerated disease progression. In the current study, we identified sociodemographic, socioeconomic, and health-related profiles of Black/African American women with SLE and examined prospective associations with organ damage accrual over a 2-year period.

Methods

Sample

The Black Women's Experiences Living with Lupus (BeWELL) Study enrolled 438 participants living in metropolitan Atlanta, GA, from May 2015 to April 2017.[14] All participants had validated SLE based on criteria set by the American College of Rheumatology, and were recruited largely from a population-based registry with supplemental sampling via regional clinics, labs, and providers. The BeWELL sample is characterized by varying levels of disease severity and socioeconomic resources. There were three main waves of data collection (baseline, 1-year follow-up, and 2-year follow-up). Data were collected between May 2015 and February 2019. Participant engagement was high, with more than 95% of survivors (n deceased = 19) completing all three waves.

Variables

Baseline measures of demographic, socioeconomic, and health characteristics were used in the latent profile analysis. Continuous measures were age in years, calculated using participant date of birth; disease duration in years, based on self-reported month and year when they were diagnosed, how long ago they were diagnosed, or their age at diagnosis (whichever was volunteered); and income-to-poverty ratio, measured continuously and calculated as self-reported annual household income before taxes divided by the poverty threshold (based on household size and number of children).[25] Categorical variables included relationship status (married or marriage-like; romantic relationship; divorced, widowed, or separated; and single), work status (full-time, part-time, out of labor force, and unable to work due to health or disability), insurance status (private, public, uninsured), and educational attainment (less than high school, high school graduate or equivalent, some college, and college degree).

SLE-related measures included disease activity and organ damage. Disease activity was measured continuously using the Systemic Lupus Activity Questionnaire (SLAQ), a validated self-report instrument designed for epidemiological research that assesses SLE symptoms in the past 3 months, such as skin rashes, fatigue, fever, and joint swelling. Possible scores range from 0 (no disease activity) to 44 (high disease activity). The Brief Index of Lupus Damage (BILD) assessed damage across 12 organ systems due to SLE.[26,27] The BILD is a validated, self-report tool used in clinical and epidemiological SLE research, with greater damage scores shown to predict quality of life, disability, and mortality.[26,27] Items assessed cumulative and irreversible damage to an organ or organ system since the onset of SLE which has been present for at least 6 months. Possible scores range from 0 to 30, with higher scores indicating greater organ damage. Self-reported SLE medication use at baseline was assessed in-person through an interviewer-administered questionnaire.[14] Prior to their interview, participants were reminded to bring information on their current SLE medication use. Trained interviewers also went through a checklist of SLE medications with each participant. In the current study, SLE medication use was scored dichotomously (yes vs no) for the following: glucocorticoids (e.g., prednisone, medrol, methylprednisolone), hydroxychloroquine, and other immunosuppressants (e.g., methotrexate, cyclophosphamide, cyclosporine, mycophenolate, dapsone, azathioprine, belimumab, rituximab).

We examined time to first accrual of new organ damage and cumulative damage accrual over two annual assessments. New organ damage was assessed as an increase in BILD score since baseline with death considered new organ damage. Time to new organ damage was calculated as the number of years between the date of baseline assessment and the date of assessment when new organ damage was identified. For participants that died before their first annual assessment, time to event was coded as 1 year. For participants that died between their first and second annual assessment, time to event was calculated as the time between baseline and the first annual assessment, plus 1 year. The BeWELL Study tracked mortality through the duration of data collection, including for participants who had already completed their final assessment. Accordingly, time to event for participants that did not experience new organ damage across two annual assessments but died after their final assessment was calculated as the number of years between baseline and their date of death.

A count variable for cumulative organ damage was calculated as the difference in BILD scores between baseline and the final completed assessment. No damage accrual or death was scored as 0; a one unit increase in BILD was scored as 1; a 2 unit increase in BILD was scored as 2; an increase in BILD of 3 or more was scored as 3 due to few participants with an increase in BILD of 4 or more units. Death was scored as 4.

Analytic plan

We used latent profile analysis (LPA) to identify subgroups or profiles of participants in the BeWELL Study based on sociodemographic, socioeconomic, and health characteristics. LPA is a exploratory modeling approach to group participants who have similar characteristics across various sociodemographic, clinical, and treatment-related domains.[28,29] It is useful for identifying complex patterns in observed characteristics that represent nuanced latent constructs or hidden clinical phenotypes.[29] The probability of each participant belonging to a specific subgroup is calculated using maximum likelihood estimation, and participants are assigned to their most likely subgroup (i.e., highest probability of belonging).[28] If model entropy is high (>0.8) and suggests clear distinction between subgroups, the resulting profiles can then be used as a predictor or outcome variable in an auxiliary model.

The best-fitting solution in LPA was determined by empirical fit indices as well as model parsimony.[30] Models of increasing profile size were used to compare fit indices of BIC and aBIC (lower is better), entropy (values closer to 1 indicate good fit), and the bootstrapped likelihood ratio test (BLRT, which statistically compares the fit of *k* vs *k*-1 profile solutions ($p < .05$ indicates a significantly better fit to the data).[28] We also examined relative gains in model fit through elbow plots of BIC values, and considered theoretical and conceptual interpretations of identified profiles.[28] After identifying a best-fitting solution, posterior probabilities and the most likely profile assignment were used for survival analyses. ANOVAs and chi-square tests identified whether LPA indicators significantly differed across profiles. Missing data for sociodemographic, socioeconomic, and SLE-related indicator variables was minimal (<1%) and accounted for using full-information maximum likelihood.

Next, we examined whether sociodemographic profiles were associated with the time to first new organ damage and cumulative organ damage accrual. Non-deceased

participants lost to follow-up after baseline (n = 11) were excluded from analyses resulting in a final analytic sample size of 427. Time to event data was interval-censored for participants who experienced new organ damage because the exact timing of damage accrual was unknown. For each participant *i* that experienced new organ damage, a response interval *(Li, Ri)* was calculated, where *Li* is the last follow-up time at which the event had not occurred, and *Ri* is the last follow-up time immediately after the event. Participants were right censored if they did not experience damage accrual or death. Survival functions were estimated for each profile and comparisons were made using generalized log-rank statistics. Proportional hazard regression models were then fit to examine the hazard function of each profile.

We also examined associations between sociodemographic profiles and the amount of new organ damage over 2 years using Poisson regression. Organ damage accumulation was modeled continuously using the following categories: no increase; BILD score increases of 1, 2 and 3 or more; and death. Estimates were calculated in referent to Higher SES with Controlled SLE participants. LPA was performed in Mplus and survival analyses were performed in SAS 9.4.

Results

On average, participants were 46.84 years old (SD = 12.31) at baseline and had been living with SLE for 15.95 years (SD = 10.37). Approximately 42% of the sample experienced damage to a new organ or organ system over the follow-up period (M = 0.68, SD = 0.99, range 0–5).

Latent profile analysis

Results of the LPA indicated a 4-profile solution best fit the data (Table S1). Profile sociodemographic characteristics are presented in Table 1. The first profile was characterized by indicators of low socioeconomic standing, moderate educational attainment, and moderate SLE severity with uncontrolled disease activity. Relative to other profiles, participants assigned to Profile 1 were younger (M = 39.61, SD = 9.64) with a shorter disease duration (M = 10.62 years, SD = 6.34), and had the lowest income-to-poverty ratio (M = 1.07, SD = 0.61). Most participants in Profile 1 reported being unable to work due to SLE (69.90%), not having a college degree (86.22%), and having public insurance (70.92%). These participants also had high disease activity (M = 17.22, SD = 7.92), moderate levels of organ damage (M = 2.41, SD = 2.50), and high rates of glucocorticoid (72.96%) and hydroxychloroquine (81.63%) use. Profile 1 represents the largest of the sample (44.75%) and was named "Younger/Lower SES with Uncontrolled SLE."

The second profile had similar low socioeconomic characteristics as Profile 1, but participants in Profile 2 were older (M = 59.07, SD = 7.62) and had been living with SLE the longest (M = 26.10, SD = 9.86). Participants assigned to Profile 2 had the most severe SLE, with high disease activity (M = 17.14, SD = 7.33) and the highest levels of organ damage (M = 3.75, SD = 2.49) at baseline compared to other profiles. Profile 2 had a higher income-to-poverty ratio compared to Profile 1, but is still characterized as living near poverty (M = 1.55, SD = 0.76). Most participants in Profile 2 were unable to work (82.24%), had public insurance (76.64%), and did not have a college degree (82.24%). These participants also reported lower use of glucocorticoids, hydroxychloroquine, and immunosuppressants compared to other profiles. About 23.29% of the sample (n = 107) is represented in Profile 2, which we named "Older/ Lower SES with Uncontrolled SLE."

Profile 3 is characterized as working-class with low disease severity and moderate socioeconomic resources. Most participants in Profile 3 were working (93.03%), either full-time (77.91%) or part-time (15.12%), and had a significantly higher income-to-poverty ratio (M = 2.48, SD = 0.96) than the first two profiles. Black/ African American women assigned to Profile 3 were educated (92.24% reported at least some college with 58.82% having obtained a college degree) and most likely to have private insurance (94.19%). Baseline levels of organ damage were lowest for Profile 3 (M = 1.49, SD = 1.61), which also had lower levels of disease activity (M = 10.65, SD = 6.55) than Profiles 1 and 2, and high use of hydroxychloroquine (79.07%). About 19.63% of the sample was assigned to Profile 3, which we named "Mid-SES with Controlled SLE."

The final profile is distinguished by high levels of socioeconomic resources, with the highest income-to-poverty ratio (M = 5.91, SD = 1.14) and educational attainment (57.14% had a college degree). Most participants in this profile worked full-time (65.31%) and had private insurance (77.55%). Profile 4 is also described by moderate levels of SLE severity at baseline, with lower disease activity (M = 10.04, SD = 626) then Profiles 1 and 2, and less organ damage (M = 2.33, SD = 2.95) than Profile 2. This final profile represents the smallest of the sample (11.19%) and was named "Higher SES with Controlled SLE."

Table 1. Sample characteristics by sociodemographic profiles in the BeWELL study (n = 438).

	BeWELL sample (n = 438)	Younger/Lower SES with uncontrolled SLE (n = 196)	Older/Lower SES with uncontrolled SLE (n = 107)	Mid-SES with controlled SLE (n = 86)	Higher SES with controlled SLE (n = 49)		
Variables	**M (SD) or n (%)**	**M (SD) or n (%)**	**M (SD) or n (%)**	**M (SD) or n (%)**	**M (SD) or n (%)**	**F or χ2**	**Tukey**
Age	46.84 (12.31)	39.61 (9.64)	59.07 (7.62)	45.50 (10.90)	51.38 (9.64)	102.12***	2>4>3>1
Years since diagnosis	15.95 (10.37)	10.62 (6.34)	26.10 (9.86)	14.74 (9.12)	17.22 (10.07)	80.52***	2>4>1; 2>3>1
Disease activity	15.11 (7.97)	17.22 (7.92)	17.14 (7.33)	10.65 (6.55)	10.04 (6.26)	26.42***	1>3; 1>4; 2>3; 2>4
Organ damage	2.55 (2.52)	2.41 (2.50)	3.75 (2.49)	1.49 (1.61)	2.33 (2.95)	14.79***	2>4; 2>1>3
Relationship status (*n, %*)						46.10***	
Married/Marriage-like	198 (45.21)	83 (42.35)	48 (44.86)	37 (43.02)	30 (61.22)	1.97	–
Romantic relationship	27 (6.16)	15 (7.65)	2 (1.87)	8 (9.30)	2 (4.08)	2.01	–
Divorced/Widowed/Separated	96 (21.69)	28 (14.29)	43 (40.19)	17 (19.77)	7 (14.29)	10.50***	2>3; 2>4; 2>1
Single	118 (26.94)	70 (35.71)	14 (13.08)	24 (27.91)	10 (20.41)	6.63***	1>2
Income-poverty ratio	2.00 (1.68)	1.07 (0.61)	1.55 (0.76)	2.48 (0.96)	5.91 (1.14)	496.96***	4>3>2>1
Work status (*n, %*)						254.30***	
Full time	125 (28.54)	25 (12.76)	1 (0.93)	67 (77.91)	32 (65.31)	120.65***	3>1>2; 4>1>2
Half time	54 (12.33)	32 (16.33)	7 (6.54)	13 (15.12)	2 (4.08)	3.35*	–
Out of labor force	22 (5.02)	2 (1.02)	11 (10.28)	1 (1.16)	8 (16.33)	10.10***	2>1; 2>3; 4>1; 4>3
Unable to work	237 (54.11)	137 (69.90)	88 (82.24)	5 (5.81)	7 (14.29)	88.17***	2>1>3; 2>1>4
Insurance (*n, %*)						247.13***	
Private	157 (35.84)	20 (10.20)	18 (16.82)	81 (94.19)	28 (77.55)	170.93***	3>4>1; 3>4>2
Public	233 (53.20)	139 (70.92)	82 (76.64)	4 (4.65)	8 (16.33)	80.42***	1>3; 1>4; 2>3; 2>4
None	48 (10.96)	37 (18.88)	7 (6.54)	1 (1.16)	3 (6.12)	8.52***	1>2; 1>3; 1>4
Education (*n, %*)						98.10***	
< High School	37 (8.47)	17 (8.67)	17 (15.89)	1 (1.18)	2 (4.08)	5.05**	2>3
High school	79 (18.08)	51 (26.02)	20 (18.69)	5 (5.88)	3 (6.12)	7.61***	1>4; 1>3
Some college	197 (45.08)	101 (51.53)	51 (47.66)	33 (34.12)	16 (32.65)	3.77*	1>3
College degree	124 (28.38)	27 (13.78)	19 (17.76)	51 (58.82)	28 (57.14)	34.35***	4>1; 4>2; 3>1; 3>2
Glucocorticoid use (*n, %*)	224 (55.71)	143 (72.96)	47 (43.93)	35 (40.70)	19 (38.78)	43.21***	1>2; 1>3; 1>4
Hydroxychloroquine use (*n, %*)	319 (72.83)	160 (81.63)	58 (54.21)	68 (79.07)	33 (67.35)	28.87***	1>2; 3>2
Other immunosuppressant use (*n, %*)	195 (44.52)	110 (56.12)	29 (27.10)	36 (41.86)	20 (40.82)	24.34***	1>2

Note: *p < .05, **p < .01, ***p < .001. Profile numbers presented for Tukey tests correspond to 1 = "Younger/Lower SES with Uncontrolled SLE" and so forth, from left to right.

Table 2.

Results of proportional hazard and Poisson regression models examining associations between profile membership and time to first damage accrual and cumulative damage accrual in the BeWELL study (n = 427).

Proportional hazard models **Profile comparison**	Hazard ratio	95% CI
Younger/Lower SES with Uncontrolled SLE vs Higher SES with Controlled SLE	1.56	(0.92, 2.67)
Older/Lower SES with Uncontrolled SLE vs Higher SES with Controlled SLE	2.41	(1.39, 4.19)
Mid-SES with Controlled SLE vs Higher SES with Controlled SLE	1.01	(0.54, 1.88)

Poisson regression models **Profile**	**b (SE)**	**95% CI**
Younger/Lower SES with Uncontrolled SLE	0.72 (0.23)	(0.27, 1.17)
Older/Lower SES with Uncontrolled SLE	0.98 (0.24)	(0.52, 1.44)
Mid-SES with Controlled SLE	0.10 (0.27)	(−0.43, 0.62)
Higher SES with Controlled SLE	(ref)	(ref)

Note: proportional hazard models examined the time to first new organ damage accrual. Poisson regression models examined the amount of new organ damage accrual over the 2-year study period.

Survival analyses

Results of generalized log-rank tests indicate that overall survival rates differed across all four profiles (χ^2 = 18.83, p < .0001). Results of proportional hazards regression models fit to the interval-censored data are presented in Table 2. Participants in the "Older/Lower SES with Uncontrolled SLE" profile had greater risk of organ damage accrual compared to the "Higher SES with Controlled SLE" profile (HR = 2.41; 95% CI = 1.39, 4.19). "Younger/Lower SES with Uncontrolled SLE" participants were also at increased risk of new organ damage, although this estimate did not reach statistical significance (HR = 1.56; 95% CI = 0.92, 2.67). Participants in the "Mid-SES with Controlled SLE" profile experienced similar risk of organ damage accrual as the "Higher SES with Controlled SLE" profile (HR = 1.01; 95% CI = 0.54, 1.88).

Poisson regression models examined associations between profiles and the amount of organ damage accrual, measured continuously in the following categories: no increase; BILD score increases of 1, 2 and 3 or more; and death (Table 2). Results indicate that compared to "Higher SES with Controlled SLE" participants, greater organ damage accrual was most strongly associated with participants in the "Older/Lower SES with Uncontrolled SLE" profile (b = 0.98, SE = 0.24, 95% CI = 0.52, 1.43), followed by those in the "Younger/Lower SES with Uncontrolled SLE" profile (b = 0.72, SE = 0.23, 95% CI = 0.27, 1.17).

Sensitivity analyses

Separate sensitivity analyses were conducted among survivors and the deceased. Results of proportional hazard models among survivors only (n = 403) were consistent with models that included deceased participants (Table S2). Poisson regression estimates among survivors were also consistent with full-sample models (Table S2), except for "Younger/Lower SES with Uncontrolled SLE" participants, whose estimate decreased in magnitude and statistical significance (b = 0.38, SE = 0.24, 95% CI = −0.09, 0.84).

Twenty-four participants died during the study period. Most (n = 15; 62.5%) were "Older/Lower SES with Uncontrolled SLE," followed by eight participants (33.3%) in the "Younger/Lower SES with Uncontrolled SLE" profile. Only 1 deceased participant (4.2%) was classified as "Mid-SES with Controlled SLE" and none were "Higher SES with Controlled SLE," thus precluding reliable regression estimates for both profiles. Results of proportional hazard and logistic regression models comparing "Older/Lower SES with

Uncontrolled SLE" and "Younger/ Lower SES with Uncontrolled SLE" participants indicate no difference in the timing of death (HR = 1.01; 95% CI = 0.40, 2.57), nor the likelihood of death over the study period (OR = 1.00, 95% CI = 0.41, 2.43).

We also conducted supplemental analyses to explore whether baseline differences in access to care and disease management across sociodemographic profiles accounted for associations with new organ damage. Proportional hazard and Poisson regression models that control for health insurance status and glucocorticoid, hydroxychloroquine, and immunosuppressant use, revealed estimates that were consistent with those of initial models.

Discussion

The objectives of this study were to identify distinct sociodemographic profiles of Black/African American women with SLE and examine prospective associations with organ damage accrual over 2 years. Our sample is from the BeWELL Study—the largest study on the social epidemiology of SLE exclusively among Black/African American women. Examining within-group variation is important for identifying mechanisms that generate racial inequities in SLE progression and outcomes.[31] We found that the timing and extent of organ damage accrual varied by sociodemographic profiles.

Older Black/African American women with fewer socioeconomic resources and uncontrolled disease activity faced the highest risk for earlier and greater organ damage accrual over time, which could not be explained by differences in medication use or health insurance. Additionally, younger, lower SES participants with uncontrolled SLE but relatively shorter disease duration also experienced elevated risk of organ damage accrual. Interventions to reduce disease activity among socioeconomically disadvantaged Black/African American women may prevent the accumulation of organ damage and reduce racial disparities in SLE outcomes. Moreover, given existing organ damage increases the risk of new organ damage, targeted efforts to mitigate disease activity specifically among younger, lower SES Black/African American women with uncontrolled SLE may effectively prevent their transition into the higher-risk, older sociodemographic profile.[8,9] Collectively, this study adds to evidence that indicates the assessment of salient social determinants of health, including socioeconomic resources, may be useful in clinical practice for identifying subgroups at higher risk for disease progression.[32]

Our findings align with past research on socioeconomic correlates of organ damage accrual in primarily white or racially diverse SLE samples.[33] Living in poverty has been prospectively associated with increased damage accumulation and in turn, mortality; and that exiting poverty confers less damage accrual compared to those who remain in poverty.[20,34] Other research found that socioeconomic factors (education, household income, and health insurance) contributed to greater, earlier, and faster organ damage accrual experienced by Black/African American patients over 13 years since diagnosis, on average.[10] Our study advances this line of research by demonstrating heterogeneity in the critical role socioeconomic resources play in organ damage accrual among Black/African American women who experience inequitable disease burden.

Socioeconomic disadvantage can hasten organ damage accrual and SLE progression through multiple pathways. SLE activity is sensitive to psychosocial stressors, including those stemming from living in or near concentrated poverty, such as perceived neighborhood disorder and exposure to crime which have been linked to adverse health outcomes for people living with SLE.[18,35] Work disability due to SLE is also common and can have socioeconomic consequences, including lost access to employer-provided private health insurance and reduced income.[36,37] Financial strain can result in prioritization of housing, food, and other basic necessities over medication adherence and other recommendations for optimal SLE monitoring.[35] Medication non-adherence also has been found to be higher among Medicaid recipients, those with greater comorbid conditions and physical limitations, and those living in racially segregated neighborhoods.[38,39] Other research has shown that many SLE patients choose to discontinue or are non-adherent to treatment regimens due to fear of adverse side effects, lack of efficacy, perceived lack of respect or compassion from providers, depression, and increasing cost and complexity of care related to longer disease duration.[40–44] Access to care has also been shown to be lowest among older SLE patients of low socioeconomic position, and many high-risk patients often do not receive the persistent preventative care they require.[45,46] Together, these factors may partially explain the lower medication use we observed among Older/Lower SES with Uncontrolled SLE participants.

More than two-thirds of Black/African American women in our sample were assigned to profiles characterized by lower levels of education, higher rates of work disability, no health insurance, and household income levels at or near

the federal poverty threshold. We found that these participants, particularly those who were older, faced the greatest risk of organ damage accrual. In contrast, one-third of participants lived above the poverty threshold and had controlled disease activity faced lower risk of organ damage accrual. Notably, only 11% of the sample was classified as relatively affluent with controlled SLE, and the low prevalence of socioeconomically disadvantaged participants with controlled SLE precluded our ability to identify this specific subgroup. Our findings are in line with other research that indicates Black/African American women with SLE report high levels of socioeconomic disadvantage.[10,47,48]

According to ecosocial theory, Black/African American women living with SLE face greater disease burden because of underlying social inequities generated and reinforced by historical, sociopolitical, and economic factors across time, space, and levels of society.[49] For instance, higher rates of poverty among Black/Africans Americans are directly linked to historical (e.g., redlining) and contemporary (e.g., racial discrimination in employment) forms of structural racism that reduce opportunities for upward economic mobility.[22,23] Beyond material socioeconomic resources, living in poverty also confers exposure to additional risk factors for SLE progression, such as neighborhood stressors and reduced access to health promoting resources via racial residential segregation.[18,35] Ecosocial theory posits that exposure to poverty and other material and psychosocial hazards become embodied over time, thus accelerating SLE progression and shaping patterns in the population-level distribution of disease burden.[49] Accordingly, our study contributes to a growing body of research that indicates racial inequities in SLE progression and outcomes are shaped by social factors, particularly those that are generated and reinforced by racism.[14–18,50]

This study has several limitations. We are unable to infer directionality and causal relationships between disease severity and socioeconomic resources. It is possible that few socioeconomic resources exacerbate SLE activity and damage; as well as the converse, that greater SLE severity at baseline results in adverse socio-economic consequences, such as work loss and poverty. Although analyses account for SLE disease severity at baseline, we were unable to account for the duration of uncontrolled disease prior to baseline, which can influence future organ damage and potentially confound estimates. Specific intervention targets among the high-risk groups cannot be deduced through our analysis (e.g., controlling disease activity and/or increasing access to socioeconomic resources); however, our person-centered analytic approach facilitated the identification of profiles which may be particularly useful for clinical practice. Future research on organ damage accrual should examine psychosocial risk and protective factors for adverse disease outcomes that are salient in the lives of Black/African American women, such as racial discrimination and social support.[14,19]

Organ damage is an important SLE outcome, yet few studies have considered sociodemographic predictors of racial inequities in damage accumulation, and those that do predominantly focus on clinical predictors among racially diverse samples.[12,33] Our study advances the social epidemiology of SLE by identifying specific subgroups of Black/African American women at greatest risk of disease progression that would otherwise go undetected using between-race study designs or variable-centered approaches that aggregate scores of individual factors. Findings from this study can be used to identify Black/African American women who may benefit the most from tailored interventions which reduce disease activity and prevent new organ damage.[32] At the structural level, policies that address underlying social inequities and facilitate upward mobility for Black/African Americans are likely to reduce racial inequities in SLE outcomes.

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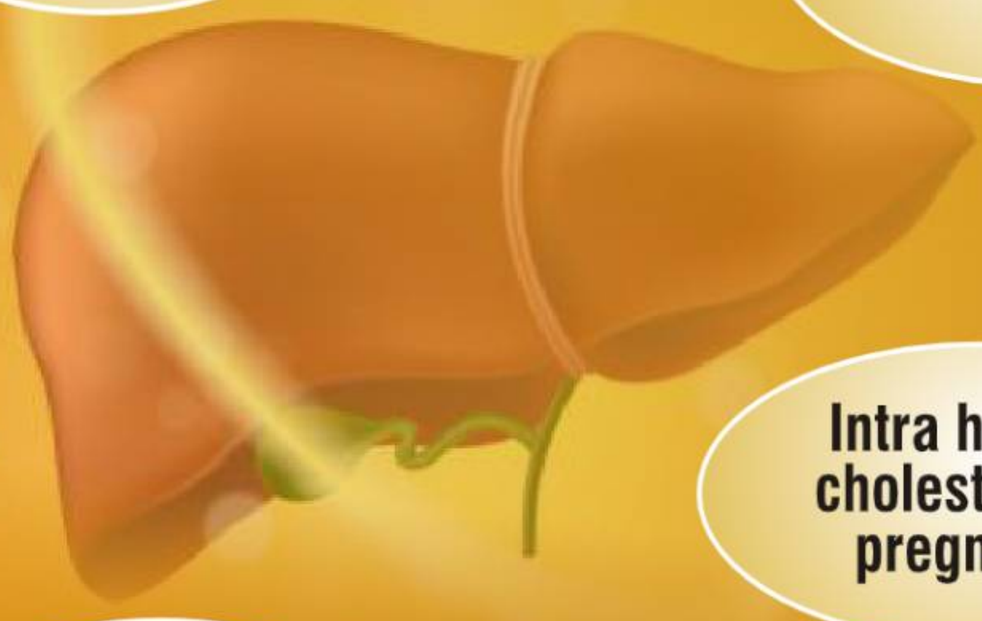
Andrographis paniculata

(standardized at 5% andrographolide)

Tablets/Syrup



Benefits:

- Improves liver functions
- Significantly suppresses HBV replication
- Protects, regulates and rejuvenates the liver
- Decreased hepato cellular injury and inflammation

Protects liver cell efficiency, correct liver cell insufficiency.

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Ceftriaxone Sodium USP Equivalent to Ceftriaxone 1g + Tazobactam Sodium Equivalent to Tazobactam 125mg

Extending the Power of Ceftriaxone to Combat ESBLs

Vs. Ceftriaxone alone

- Leads to drop in resistance from 88% to 4%[1]
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- 4 times faster recovery[2]
- Excellent therapeutic choice[1]

Benefits

- Effective against Gram -ve bacilli and ESBL producers[3,4]
- Empirical therapy[5]
- Reduces severity in life threatening conditions[5]
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Indications:

Nosocomial Pneumonia	Catheter induced infections
Complicated Urinary Tract Infection	Contaminated and Clean Contaminated Surgical Prophylaxis
Septicemia	

Dosage:

Adults: 1.125 to 2.250 grams once daily (or equally divided doses, twice daily), not to exceed 4.5 grams/day

Pediatric patients: 50 to 75 mg/kg, once daily (or equally divided doses, twice daily), not to exceed 2.250 grams/day

For preoperative use: a single dose of 1.125 gm, ½ to 2 hours before surgery

Please refer Prescribing information for more details on dosage

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