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REVIEW

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Pathogenic signature of invasive non-typhoidal *Salmonella* in Africa: implications for vaccine development

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ABSTRACT

Invasive non-typhoidal *Salmonella* (iNTS) infections are a leading cause of bacteremia in Sub-Saharan Africa (sSA), thereby representing a major public health threat. *Salmonella* Typhimurium clade ST313 and *Salmonella* Enteriditis lineages associated with Western and Central/Eastern Africa are among the iNTS serovars which are of the greatest concern due to their case-fatality rate, especially in children and in the immunocompromised population. Identification of pathogen-associated features and host susceptibility factors that increase the risk for invasive non-typhoidal salmonellosis would be instrumental for the design of targeted prevention strategies, which are urgently needed given the increasing spread of multidrug-resistant iNTS in Africa. This review summarizes current knowledge of bacterial traits and host immune responses associated with iNTS infections in sSA, then discusses how this knowledge can guide vaccine development while providing a summary of vaccine candidates in preclinical and early clinical development.

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1. Introduction

There is growing awareness that foodborne diarrheal agents, such as non-typhoidal *Salmonella* (NTS), pose a serious threat to public health in resource-limited settings.[1] NTS are facultative, anaerobic, intracellular *Enterobacteriaceae* belonging to the *Salmonella enterica* (*S. enterica*) species.[2] They are commonly transmitted by the consumption of contaminated food or water and have a broad host-range.

Following ingestion, a proportion of the infectious *Salmonella* load survives the acidic pH of the stomach and competition with the gut microbiota. *Salmonella* can penetrate the intestinal epithelial barrier by invading non-phagocytic cells, such as enterocytes, or, preferentially, microfold cells (M cells) of Peyer's Patches, through the expression of a multiprotein needlelike apparatus (the Type III Secretion System (T3SS) encoded by *Salmonella* Pathogenicity Island 1 (SPI-1-T3SS)), which is used as a conduit to translocate effector proteins into the host cell cytoplasm. Gut luminal *Salmonella* can also be taken up by dendritic cells (DCs) or be phagocytized by CD18+ intestinal macrophages.[3] Virulent *Salmonellae* use several strategies to elude the immune system and promote bacterial growth, including interference with the DC-mediated antigen presentation process or modification of the phagosome environment.[3]

Salmonella-containing macrophages and DCs constitute a vehicle for systemic dissemination, as these cells can rapidly migrate[3] and spread through the bloodstream to extra-intestinal sites, such as the spleen and bone marrow.[4] However, in otherwise healthy individuals, NTS infections remain localized in the small intestine and colon[3] and mostly cause a self-limiting enterocolitis, which is commonly associated with secretory diarrhea, vomiting and abdominal pain. Hepatomegaly, splenomegaly or gastrointestinal complications (which may include cholecystitis, pancreatitis and appendicitis) are not frequently observed in NTS patients.[5]

Recent estimates report that *Salmonella* enterocolitis resulted in 95.1 million cases and 50,771 deaths globally in 2017.[6] In countries with medium-to-high socio-demographic development, only a small proportion (1–3 cases per 100,000 person-years) of the population develops an invasive *Salmonella* infection, and this is more likely to occur in children, the elderly and immunocompromised individuals.[6] By contrast, NTS species are often responsible for life-threatening systemic infections in low-income settings, where poor sanitation, lack of proper diagnostic tools, malnutrition and other comorbidities can further exacerbate the outcome of the disease.[7,8]

According to current incidence data, invasive non-typhoidal *Salmonella* (iNTS) infections particularly haunt Africa. Of the estimated 535,000 cases of iNTS disease and 77,500 deaths that occurred globally in 2017, the highest incidence (34.5 cases per 100,000 person-years) was observed in Sub-Saharan Africa (sSA).[6] An average case-fatality rate of 20.6% was recently reported among iNTS patients in sSA, with peaks up to 72% in people presenting infections caused by the human immunodeficiency virus (HIV).[9]

The typical clinical presentation of iNTS disease in Africa is a nonspecific febrile systemic illness, which is often accompanied by respiratory symptoms and hepatosplenomegaly. For this reason, iNTS infections can be confused with comorbidities commonly reported in sSA, such as malaria and pneumonia. African patients with iNTS infections also display the features of other concurrent conditions which are frequently

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observed in sSA, such as anemia, malnutrition, and advanced HIV illness. Diarrhea is not a predominant symptom of iNTS disease in Africa.[5]

The most common invasive *S. enterica* strains identified in African regions since 1966 belong to the *Salmonella* serovar Typhimurium (*S.* Typhimurium) and the *Salmonella* serovar Enteritidis (*S.* Enteriditis),[9] although other serovariants, such as *Salmonella* Dublin (*S.* Dublin), *Salmonella* Concord (*S.* Concord), *Salmonella* Stanleyville (*S.* Stanleyville) and *Salmonella* Isangi (*S.* Isangi) have been reported in sSA.[10]

During the last 5 decades, several countries of sSA have experienced multiple outbreaks associated with a multidrug-resistant (MDR) iNTS classified as *S.* Typhimurium sequence type (ST) 313 (ST313).[11] Whole genome sequencing (WGS) of iNTS isolates from sSA has identified two closely related, but genetically distinct, ST313 lineages, which emerged in the 1960 s (lineage 1) and late 1970 s (lineage 2).[12] It is supposed that around 20 years ago, as a result of the acquisition of chloramphenicol resistance, ST313 lineage 2 gained an evolutionary advantage over lineage 1 and, consequently, clonally replaced it. Additionally, the rapid expansion of lineage 2 MDR ST313 strains across sSA seems to have occurred in parallel with the spread of HIV epidemics,[12] suggesting that these iNTS clones may have adapted to a specific human niche characterized by an immunocompromised population and the extensive use of antibiotics.

WGS studies have also identified two related, but geographically and genetically distinct, *S.* Enteriditis lineages that emerged over the last 90 years in sSA, and which are referred to as the "Western African" (WEA) and "Central/Eastern African" (CEA) clades.[13] Like the ST313, these clades almost exclusively cause invasive disease in humans and display resistance to commonly used antibiotics.[13]

The WEA, CEA and ST313 clades share some virulence features with invasive host-restricted *Salmonella* serovars such as *Salmonella* Typhi (*S.* Typhi) and *Salmonella* Gallinarum (*S.* Gallinarum), including genome degradation, the presence of multidrug resistance elements, and a unique prophage repertoire.[11,13] Moreover, like the human-restricted *S.* Typhi, iNTS isolated in sSA rarely cause infections associated with gastrointestinal symptoms, such as inflammatory diarrhea.[10,14,15] To date, an animal reservoir for iNTS has not been identified,[16] whereas asymptomatic carriage has recently been described in humans.[17,18] These observations corroborate the hypothesis that African NTS serovars are increasing their potential for systemic dissemination in humans and can be spread from person to person, in addition to the classic zoonotic transmission.

Along with host susceptibility,[15,19] the intrinsic pathogenic signature of African iNTS lineages might explain the high rates of invasive non-typhoidal salmonellosis observed in sSA. Investigating the host- and pathogen-related factors that predispose to iNTS disease may aid the design of efficacious prevention strategies, which are not yet available for implementation in humans.

This review summarizes current knowledge of the bacterial features and host susceptibility factors that contribute to the development of NTS bacteremia in sSA. We also provide a summary of iNTS vaccine candidates currently in the early developmental stage and discuss how information gathered through the study of host-pathogen interactions can be used in iNTS vaccine research.

2. iNTS features associated with invasive infections in Africa

2.1. Multidrug resistance

One of the main drivers of NTS bloodstream infections in Africa is the emergence of MDR iNTS,[11,20–22] which can acquire determinants of antimicrobial resistance via plasmid conjugation. The spread of plasmids that harbor both virulence and drug-resistance elements ("Virulence-Resistance Plasmids" -VRP-) has increased alarmingly in recent years within NTS serovars capable of systemic dissemination in humans, such as *Salmonella* Choleraesuis (*S.* Choleraesuis), *S.* Dublin, *S.* Enteriditis and *S.* Typhimurium.[23]

A clinical gastroenteritis-causing MDR *S.* Enteriditis isolate recovered in Spain in 2003 was found to carry one of these VRP, and was dubbed pUO-SeVR1.[24] This is a derivative of the common *S.* Enteriditis-associated plasmid pSEV and carries the *spv* virulence locus, together with genetic elements that confer resistance to a range of widely used drugs, such as ampicillin, chloramphenicol, streptomycin, sulfonamides, tetracycline and trimethoprim.[24] pUO-SeVR1 also shows accumulation of insertion sequences,[25] a feature which is regarded as an evolutionary step toward host restriction.[26] The presence of pUO-SeVR1–like plasmids in the WEA and CEA clades,[13] as well as in *S.* Enteriditis isolates from patients of African origin or travelers to Africa,[24,27] has been associated with MDR invasive infections in humans.

In the *S.* Typhimurium ST313 lineage 2, the MDR-associated genotype is mostly encoded by integrons inserted in the pSLT-BT plasmid. pSLT-BT is closely related to pSLT, a virulence plasmid containing the *spv* genes involved in systemic infection by *S.* Typhimurium in mice.[11] pSLT-BT includes a Tn21-like mobile element harboring multiple resistance loci which resemble those identified in pUO-SEVR1-like plasmids.[11,21,24,28] The MDR spectrum of ST313 is further enriched by the presence IncHI2 plasmids.[29] IncHI2 plasmids harbored by iNTS strains isolated in Kenya,[29,30] Malawi,[21] and the Democratic Republic of Congo (DRC)[31] have been seen to carry resistance to extended-spectrum β-lactams (ESBLs), including ceftriaxone, a third-generation cephalosporin regarded as one of the last options in the case management of iNTS infections. The presence of ESBL resistance elements has also been reported in MDR *S.* Typhimurium isolates from the DRC,[32] Burkina Faso,[33] Senegal[34] and Nigeria.[35]

Along with ceftriaxone, fluoroquinolones (FQ) are one of the few classes of antibiotics currently recommended for use against invasive *Salmonella* infections.[36,37] Although FQ resistance is still rarely reported in iNTS serovars, careful surveillance is necessary, since both FQ-resistant invasive *S.* Typhimurium and *S.* Enteriditis have been observed in African countries.[13,28–30,32,34,37,38] This highlights the need not only for prudent use of antimicrobials, but, most importantly, for the implementation of preventive strategies against iNTS.

2.2. Genetic differences that influence the pathogenesis of iNTS and NTS strains

The *S.* Typhimurium ST313 clade is phylogenetically distinct from common gastroenteritis-associated *S.* Typhimurium lineages such as ST19. Some features, including genome degradation and the presence of novel prophages carrying virulence genes, distinguish ST313 isolates from ST19 strains, and may favor the spread of ST313 toward systemic sites.

Sequence analysis of the representative African ST313 lineage 2 strain D23580 isolated in Blantyre (Malawi)[11] revealed the presence of two strain-specific prophages, named Blantyre Prophage 1 (BTP1) and Blantyre Prophage 5 (BTP5),[11,39] which are absent in the ST19 clade[39] or in ST313 variants found in the United Kingdom (UK).[40] One BTP1 gene, *st313-td*, has been associated with increased ST313 replication within macrophages, a key feature of the development of systemic *S.* Typhimurium infections.[41] Another BTP1 element, the *gtrAC* operon, has been found to encode a glycosyltransferase which modifies the composition and length of the O-antigen (OAg),[42] the main polysaccharide component of the Gram-negative lipopolysaccharide (LPS). The OAg is well known to be involved in resistance to antibody-mediated killing,[39,42–45] and longer OAg chains have been associated with increased iNTS serum resistance.[43] Since the *gtrC* operon has proved to play a role in the retention of full-length OAg,[42] and deletion of *st313-td* has been associated with virulence attenuation in a mouse model of systemic NTS infection,[41] it is likely that these genetic elements contribute to the enhanced ability of African ST313 to persist systemically within the host.

Loss of coding sequences and the consequent formation of pseudogenes (PSD) are associated with the adaptation of *Salmonella* to new host niches, and may facilitate systemic infection. The accumulation of PSD is more frequent in host-specialized or -adapted *Salmonella*, such as *S.* Gallinarum (more than 300 PSD),[46] *S.* Typhi and *Salmonella* Paratyphi A (*S.* Paratyphi A) (204 and 177 PSD, respectively),[47,48] *Salmonella* Paratyphi C (*S.* Paratyphi C) and *S.* Choleraesuis (about 160 PSD),[49] than in generalist serovars like *S.* Typhimurium[50] and *S.* Enteriditis,[46] suggesting that the frequency of gene-inactivating mutations may be linked to host adaptation.

The genome of D23580 carries at least 77 PSD, 23 of which are strain-specific.[11,51,52] One of the most recently discovered is the *sseI* pseudogene. When functional, *sseI* encodes the SseI effector of the *Salmonella* Pathogenicity Island 2-induced T3SS (SPI-2-T3SS), a kind of T3SS which is expressed by *Salmonella* following internalization within cells such as macrophages and DCs. The SseI effector has been found to inhibit DC migration by altering chemotaxis.[53] In a murine infection model, pseudogenization of *sseI* resulted in rapid CD11b+ DC-mediated migratory hyper-dissemination toward draining lymph nodes.[54] Additionally, *sseI* is either absent or inactivated in invasive host-restricted or highly adapted *Salmonella* serovars.[46,54–57] This further emphasizes the importance of gene inactivation as a strategy for promoting bacterial spread toward extra-intestinal sites.

In NTS, the *fliC* gene encodes phase 1 flagellin FliC, a surface antigen which plays a role in motility and cell invasion, but which is also a target of innate and adaptive immune responses. Cummings et al.[58] demonstrated that, while extracellular *S.* Typhimurium abundantly expresses FliC, intracellular *Salmonella* at systemic sites down-regulates *fliC* expression below the threshold required for the activation of T cell responses, in order to evade host defenses. Repression of flagellin synthesis has been associated with invasive *Salmonella* infections, such as those caused by the host-restricted *S.* Dublin[59] and *S.* Typhi.[60] Ramachandran and colleagues[61] demonstrated that clinical ST313 with attenuated flagella isolated in Mali were phagocytosed more efficiently by murine J774 macrophages and were less susceptible to macrophage killing than ST19. Carden et al.[62] found that, in comparison with ST19, inflammasome activation within macrophages was markedly reduced in the case of ST313 infection, as evidenced by decreased interleukin (IL)-1β production and caspase-1-induced macrophage death. As flagellin can trigger inflammasome activation in macrophages, and FliC production is reduced in ST313 strains,[62] it is likely that the decreased sensing of flagellar proteins by the inflammasome complex can promote the enhanced intracellular survival of African ST313 strains.

Genes involved in iron uptake and siderophore secretion within macrophages have proved to be down-regulated to a greater degree in the ST313 D23580 strain than in ST19 strains.[51] *S.* Typhimurium with defects in iron acquisition have shown enhanced ability to grow systemically in a mouse model co-infected with malaria parasites, which are known to increase intracellular iron availability.[63] This suggests that repression of genes associated with iron metabolism could be the result of niche adaptation.[51]

A single nucleotide polymorphism (SNP) which up-regulates the expression of the *pgtE* gene has recently been identified as a feature of the African ST313 D23580 strain, thereby differentiating it from ST19 strains.[64] *pgtE* encodes the outer membrane protease PgtE, which is highly expressed by *Salmonella* upon exit from macrophages, and interferes with the complement cascade. The increased production of PgtE results in reduced complement deposition on the surface of D23580, making this ST313 strain more resistant to complement-mediated serum killing.[64] The same *pgtE* mutation has been found in the *S.* Gallinarum serovar.[64] PgtE is also up-regulated in human macrophages during *S.* Typhi infection,[65] strengthening the hypothesis that increased expression of this protease is linked to an invasive phenotype and to host adaptation.

Lower *sopE2* mRNA levels have also been observed in ST313 strains than in ST19 strains.[62] *sopE2* down-regulation results in decreased expression of the SPI-1-T3SS effector protein SopE2 and has been associated with decreased invasion of epithelial cells.[62] Interestingly, *sopE2* is a pseudogene in the human-restricted *S.* Typhi[66] and shows point mutations in the invasive, avian-adapted *S.* Gallinarum.[46] In addition, deletions in the *pipD* gene (which is implicated in fluid accumulation in ileal bovine loops) have been associated with a diminished ability of ST313 lineage 2 isolates to elicit inflammation of the mammalian intestinal tract.[67]

Numerous inactivating mutations in metabolism-related genes (such as *allB*, *allP*,[11,40,51,67] *ttdA*[67] and *melR*[51]) and in genes involved in intestinal persistence (such as *ratB*[67,68] and *macAB*[40,69]) have been described in the ST313 clade but not in the ST19 clade; however, their influence on the pathogenesis of iNTS disease remains to be clarified.

Table 1. Main genetic differences and associated phenotypic changes of *S.* Typhimurium D23580 (ST313) in comparison with ST19 strains.

Gene/prophage	Genetic change(s) compared to ST19	Phenotypic change(s) compared to ST19
pSLT-BT plasmid	Insertion of a composite Tn21-like mobile element (absent in ST19) [11]	CAKSSuW resistance [11]
sseI	Pseudogene (insertion of an IS200 element) [11,40,51,54,67]	Hypermigration DCs [54]
lpxo	Pseudogene [11,40]	Unknown
fliC	Reduced *fliC* mRNA expression compared to SL1344 and DT104 [62]	Reduced NLRC4 inflammasome activation and macrophage death [62]
pgtE	SNP at the −12 position of the pgtE TSS compared to 4/74 [64]	Increased resistance to complement-mediated serum killing [64]
BPT1	Novel prophage (absent in LT2) [39]	10,000-fold greater spontaneous phage production [39]
BPT5	Novel prophage (absent in LT2) [39]	Unknown
st313-td (bstA)	Novel gene carried by BTP1 propage (absent in LT2) [41]	Increased intra-macrophage replication [41]
gtrC	Novel gene carried by BTP1 propage (absent in LT2) [39,42]	Acetylation of rhamnose residues of O-antigen [42]
iroC/iroD	Reduced expression in macrophages compared to 4/74 [51]	Unknown
allP/allB	Pseudogenes (1694 base pairs deletion) [11]	Reduced allantoin degradation [11]
ttdA	Pseudogene (nonsense mutation G-A nucleotide change) compared to SL1344 [67]	Reduced L- tartrate degradation [67]
melR	Pseudogene compared to 4/74 [51]	Inability to ferment melibiose [51,70]
ratB	Pseudogene (nonsense mutation G-A nucleotide change) compared to SL1344 [67]	Possibly reduced cecal colonization and fecal shedding[1] [68]
sopE2	Reduced *sopE2* mRNA expression compared to SL1344 and DT104 [62]	Reduced invasion of intestinal epithelial cells [62]
macB	Pseudogene [40]	Possibly reduced intestinal colonization[2] [69]
pipD	C-terminal deletion compared to LT2, SL1344 and DT104 [11,67]	Reduced enteropathogenicity in bovine ligated ileal loops [67]

CAKSSuW: A, ampicillin; C, chloramphenicol; K, kanamycin; S, streptomycin; Su, sulfonamide; W,trimethoprim. TSS: Transcriptional Starting Site.
[1]Phenotype of *ΔratB* AH12 *S.* Typhimurium strain (ST19) compared to wild-type AJB715 *S.* Typhimurium strain (ST19) in CBA/J mice challenge. studies
[2]Phenotype of *ΔmacAB* mutant strains derived from *S.* Typhimurium strain ATCC 14028 (ST19) compared to wild-type *S.* Typhimurium HA420 strain. (ST19) in BALB/c mice challenge studies

A summary of the main genetic differences and associated phenotypic changes of the *S.* Typhimurium ST313 D23580 strain, in comparison with ST19 strains, is reported in Table 1.

3. Host risk factors that increase susceptibility to iNTS disease

Extremes of age are common risk factors for invasive non-typhoidal salmonellosis globally.[6,71,72] However, a different bimodal age distribution of iNTS disease cases has been found in sSA, with adults and children aged < 5 years bearing the highest disease burden.[6,70–73] The fact that African adults are one of the most severely affected groups may be explained by the higher prevalence of HIV in the middle-aged population.[70] Indeed, together with malaria[8] and malnutrition,[74] HIV has been identified as a major predisposing factor for disseminated NTS infections. This is further emphasized by the significant association between the administration of antiretroviral therapy (ART) and the reduced occurrence of *S.* Typhimurium bacteremia in South Africa.[75]

HIV infection causes loss of CD4 + T helper 17 cells in the gastrointestinal mucosa, and therefore reduces local IL-17 levels. This leads to decreased neutrophil infiltration and promotes the spread of *Salmonella* from the gut toward systemic sites.[76] Additionally, the depletion of CD4 + T cells results in an unbalanced T helper 1 (Th1)/T helper 2 (Th2) response, with decreased secretion of Th1 cytokines such as gamma interferon (IFN-γ)[77] in favor of skewing toward a Th2 response.[78] Susceptibility to iNTS in HIV-infected African adults is also promoted by an excessive production of anti-LPS immunoglobulins G (IgG).[79] This dysregulated humoral response interferes with antibody-dependent complement-mediated killing in a concentration-dependent way[80] and probably allows *Salmonella* to establish an intracellular niche before serum bactericidal antibodies can exert their activity.[81]

Like HIV-iNTS co-infection, the concurrent presence of iNTS and malaria parasites leads to the suppression of intestinal inflammation. This attenuated immune response within the gut mucosa has been linked to an increased production of the anti-inflammatory cytokine IL-10,[82] resulting in an increased availability of intracellular iron[83] that can be exploited by *S.* Typhimurium to promote intracellular growth,[63] particularly within malaria-induced hemophagocytic macrophages (which show reduced microbicidal activity[84–86]) and neutrophils.[87,88] The systemic dissemination of iNTS is also facilitated by other malaria-induced perturbations of the immune response: *(i)* consumption of the C3 complement component, which reduces the efficiency of antibody-mediated bactericidal killing;[89] and *(ii)* inhibition of the production of circulating IL-12, a cytokine which plays an important role in the regulation of IFN-γ release, and hence in the clearance of invading pathogens.[85]

Malnutrition may increase susceptibility to invasive infections by impairing the integrity and acidity of the gastrointestinal barrier[90] and by negatively affecting the functions of the immune system. Reduced phagocytosis, neutrophil chemotaxis and bactericidal activity have been reported in children affected by protein-energy malnutrition.[91,92] Lower proportions of circulating effector CD4+ and CD8 + T cells,[93] and CD4 + T cells,[94] and the reduced ability of these latter to mount a protective memory response against infectious agents,[95] have been observed in malnourished infected children in comparison with well-nourished infected children.

Additionally, a number of genetic risk factors (reviewed in reference[19] and[96]) have been associated with increased susceptibility to iNTS disease. This particularly applies to primary immunodeficiencies, such as Chronic Granulomatous Disease (CGD), Mendelian Susceptibility to Mycobacterial Disease (MSMD) and Sickle Cell Disease (SCD).

CGD is a hereditary disorder in which impaired ROS production induces a hyper-inflammatory state that is characterized by phagocytes with defective microbicidal activity and reduced control over innate and T cell responses.[97] MSMD is a collection of inherited deficiencies that affect the IL-12/23-IFN-γ pathway, with consequent impairment of IFN-γ-mediated immunity;[98] The fact that NTS infections in patients with CGD or MSMD can be successfully treated with antibiotic therapy and IFN-γ[98] highlights the contribution of IFN-γ to immunity against *Salmonella* and the importance of a functional innate immune system in protecting against invasive *Salmonella* infections.[99]

SCD is a group of genetic blood disorders characterized by altered neutrophil activity,[100,101] impaired splenic functions,[102] reduced CD4/CD8 ratio[103] and reduced serum bactericidal activity (resulting from the impairment of the alternative complement pathway).[104] A large study involving Kenyan children with SCD revealed that 18% of bacteremia cases reported in this group were attributed to NTS species.[105] Similar results were observed in SCD patients from a Tanzanian tertiary-level hospital.[106] In Burkina Faso[107] and Cameroon[108] around 30% of bacterial infections occurring in SCD patients were associated with *Salmonella* species. Between 2010 and 2015, *S.* Typhimurium was recovered in more than 50% of Gambian SCD children presenting with an invasive bacterial infection.[109] These data further emphasize the importance of targeted immunization programs in subjects with SCD; yet no clinical trial to assess the efficacy and safety of *Salmonella* vaccines is ongoing in SCD patients.[110]

Carriage of an SNP on the *STAT4* gene was recently associated with an increased risk of iNTS bacteremia during a Genome Wide Association Study (GWAS) involving Malawian and Kenyan children with iNTS disease.[111] The presence of the *STAT4* locus rs13390936 in this population affected the ability of NK cells to produce IFN-γ following IL-12 stimulation, and resulted in decreased IFN-γ serum levels. The presence of the risk allele did not modify the proportion of IFN-γ-producing CD4 + T cells, suggesting that the *STAT4* variant enhances the probability of developing invasive non-typhoidal salmonellosis independently from the HIV status. Additionally, no association of the rs13390936 locus with malaria or malnutrition was found. This emphasizes once again the importance of IFN-γ in the response against iNTS.[111]

4. Protection against iNTS: the role of antibodies and T cell-mediated immunity

The early response against *Salmonella* relies on innate immunity within the gut mucosa. Neutrophils[112] and macrophages[113] are key players in this phase, since they produce, among other antimicrobial peptides, ROS that are involved in the respiratory burst, an essential defense mechanism for killing intracellular pathogens.[114,115] Recent studies have also shown that neutrophils are an important source of mucosal IFN-γ during *S.* Typhimurium-induced colitis in mouse models.[112] The depletion of neutrophils allows *S.* Typhimurium to grow extracellularly and increases bacterial burden in murine spleen[112] and liver,[116] suggesting that the neutrophilic compartment can curb bacterial dissemination to extra-intestinal sites.[82,116]

However, as the infection proceeds, effective immune responses against *Salmonella* depend on the generation of T cells and antibody responses.[45,99,117–119] At a later stage of infection, T cells are among the main producers of IFN-γ[120] and can delay the spread of bacteria during the intracellular phase of *Salmonella*. Additionally, $CD4^+$ T cells stimulate B cells to produce antibodies[118] which are able to control extracellular *Salmonellae* via classical/alternative complement pathways and opsonophagocytosis.[45,121]

CD4 + T helper cells seem to play a greater role in immunity against *Salmonella* infections than CD8 + T cells.[119] Loss of CD4 + T cells (e.g. as occurs in advanced HIV) is one of the primary correlates of susceptibility to NTS bacteremia[4] and can also perturb antibody responses to *Salmonella*.[79,80] African iNTS are reported to elicit bactericidal antibodies against OAg,[122] and the acquisition of antibodies to iNTS LPS OAg in Malawian children has been seen to correlate with a decline in the incidence of invasive disease,[123] suggesting that serum killing may have an important role in protecting against iNTS. Decreased levels of anti-LPS IgG and immunoglobulins A (IgA) have also been observed in CD4 + T cell-depleted mice,[124] emphasizing the importance of T cell-mediated immunity in the induction of anti-iNTS humoral responses.

The critical role of NTS-specific antibodies in preventing bacteremia is also deducible from various observations: subjects with deficiencies in the IL-12/IL-23/IFN-γ axis, despite impairment in this pathway, do not suffer fatal *Salmonella* infections, most probably because of the functional antibody activity against NTS;[125] the age-dependent decline in the occurrence of iNTS, corresponding to sequential acquisition of antibodies;[123] the relatively low incidence of iNTS disease observed in newborns, a protection probably afforded by maternal antibodies;[45,123] and the finding that anti-LPS antibodies from both HIV and non-HIV infected subjects are bactericidal *in vitro* at very low titers.[126]

These observations, along with the fact that humoral responses elicited by NTS vaccination are preserved in many iNTS-predisposing conditions,[127–129] suggest that vaccine-induced protection against iNTS should focus on the induction of both antibodies and T cell-mediated immunity.

5. iNTS vaccine development

5.1 Live attenuated vaccines

Salmonella live attenuated vaccines (LAVs) contain strains that carry attenuating mutations but maintain their immunogenic capacity. These vaccines can deliver multiple antigens that stimulate the immune system and elicit both humoral and cell-mediated responses.[130] Other advantages of *Salmonella* LAVs are their potential to induce cross-protection as well as mucosal immunity, and their convenient oral administration.[131]

While an oral live attenuated vaccine against *S.* Typhi (Ty21a) has been available for more than 2 decades, it shows moderate immunogenicity and efficacy and has not been prequalified by the World Health Organization (WHO).[132]

With regard to NTS, the only live attenuated vaccine that has entered the clinical stage is a vaccine based on *S.* Typhimurium attenuated by means of deletion of the *aroC* and *ssaV* genes (WT05).[133] The introduction of these mutations has yielded strains with defective aromatic acid biosynthesis, which are unable to properly form the SPI-2-T3SS apparatus required for the invasion of phagocytic cells. Although WT05 has displayed good tolerability and elicited high anti-LPS antibody responses in healthy human volunteers, the vaccine strains have proved to be excreted in stools for more than 3 weeks.[133] Prolonged fecal shedding of viable organisms constitutes a problem for vaccine development, as it increases the risk of food or water contamination, and consequently of transmission. For this reason, the WT05 candidate has not been tested further.

Other oral *Salmonella* LAVs based on attenuated *S.* Typhimurium and *S.* Enteriditis strains are under investigation.[134,135] Among the most recently developed are CVD 1931 (*S.* Typhimurium D65 *ΔguaBA ΔclpX*) and CVD 1944 (*S.* Enteritidis R11 *ΔguaBA ΔclpX*), which carry mutations in the *guaBA* and *clpX* genes. These mutations reduce bacterial virulence by impairing the guanine synthesis pathway and increasing the expression of flagella. Both vaccine strains have shown excellent immunogenicity after two and three immunization doses, and provide both protection in BALB/c mice following homologous challenge and cross-protection against heterologous *Salmonella* serovars.[135] The protection against mortality conferred by *S.* Enteriditis CVD 1944 exceeds 80% in mice challenged with the heterologous strain *S.* Typhimurium D65 (ST313). Similarly, CVD 1931 vaccine efficacy is high against *S.* Stanleyville, which causes sporadic antibiotic-resistant clinical cases in sSA.[135] More recently, it has been suggested that NTS LAVs carrying heterologous OAg may be effective against *Salmonella* infections, although the protection they confer is highly O-serotype-specific.[136,137]

No data are currently available on the fecal shedding of CVD 1931, CVD 1944 or OAg-based NTS LAVs. In monkeys infected with the ST313 strain *S.* Typhimurium D65, excretion of bacteria in the feces stopped 10 days post-infection, whereas in most of the rhesus macaques infected with the ST19 *S.* Typhimurium I77 fecal shedding was observed up to 18 days after infection.[138] The short-lasting stool excretion of the ST313 clade may justify the use of these organisms, rather than gastroenteritis-associated strains, for the construction of LAVs against *S.* Typhimurium. Alternatively, multiple attenuations that reduce intestinal persistence can be introduced into vaccine strains derived from commonly spread *Salmonella*. In this regard, Ghany and colleagues[139] found that introducing mutations into *hdA, misL*, or *ratB* genes could reduce the fecal shedding of *S.* Typhimurium in mice without affecting immunogenicity. Moreover, Wang et al.[140] reported that the expression of the *Salmonella* bactericidal *yncE* gene induced by oral administration of arabinose 24 hours post-vaccination with an *S.* Typhimurium-based live attenuated vaccine was able to eliminate bacteria in the murine intestinal tract, with no significant impact on anti-LPS and anti-flagellin IgG titers or on protection upon challenge.

Along with the risk of prolonged fecal shedding, the use of *Salmonella* LAVs raises additional concerns. One of these is the possibility of an *in vivo* reversion of LAVs to a wild type phenotype through the re-acquisition of deleted genes; however, the presence of double (or triple) genetically distinct mutations in the vaccine strain should prevent the risk of regaining virulence.[141] Moreover, a good balance between vaccine immunogenicity and reactogenicity must be achieved in humans, especially in immunocompromised hosts. Indeed, one of the main problems associated with LAVs is their potentially harmful effect in individuals with immune suppression; this could hamper the use of LAVs in sSA, where malaria and HIV infections are common.

A temporary loss of vaccine-mediated protection has been observed in mice co-infected with an *S.* Typhimurium live attenuated vaccine and malaria parasites.[127] This absence of protective immunity was mostly attributed to suppression of T cell effector responses and to an increased IL-10 expression.[127] While LAV responses depend on cellular immunity, the loss of T cell-mediated effector immunity would not be expected to affect responses to vaccines such as the glycoconjugates.[127] Indeed, these rely on antibody-mediated immunity as their main mechanism of action, while still requiring T cell help in order to induce immunological memory.[131]

5.2. Glycoconjugate vaccines

Protein-polysaccharide vaccines (also known as glycoconjugates) show some advantages over pure glycans in terms of vaccine-induced immunity, as they can stimulate a T cell-dependent antibody response and immune memory.[131] Additionally, they can overcome the safety issues associated with the use of LAVs.

5.2.1. Glycoconjugate vaccines against S. Typhi

Several glycoconjugate vaccines against typhoid fever have been developed in the last decade. The first typhoid conjugate vaccine (TCV) developed was based on the *S.* Typhi Vi capsular polysaccharide and the recombinant mutant of *Pseudomonas aeruginosa* exotoxin A as a carrier protein (Vi-rEPA). This vaccine displayed almost 90% efficacy in Vietnamese children aged 2 to 5 years, and was submitted for in-country licensure in China in 2013.[132] Three formulations based on the *S.* Typhi Vi polysaccharide conjugated to tetanus toxoid (TT) (Vi-TT) are currently licensed in India for use in subjects aged > 6 years. One of these, the Typbar-TCV, which was WHO-prequalified in late 2017, is being used in India and Pakistan, and is under evaluation in many countries.[132] Recently, this vaccine was found to be immunogenic and effective in reducing the burden of typhoid fever in a clinical field trial conducted in children aged 9 months to 16 years living in an endemic setting.[142] Additionally, a vaccine containing the Vi antigen conjugated with the recombinant nontoxic mutant of diphtheria toxin (CRM197) (Vi-CRM197) is currently under investigation and has proved to be safe and immunogenic in phase 2 clinical trials conducted in both endemic and non-endemic regions.[132] Furthermore, a vaccine based on the Vi polysaccharide conjugated to diphtheria toxoid (DT) (Vi-DT) has recently proved to be safe and immunogenic both in a phase 1 clinical trial involving 2- to 45-year-old

participants[143] and in a phase 2 trial conducted in children aged 6 to 23 months.[144]

The Vi-rEPA-, Vi-TT-, Vi-CRM197- and Vi-DT-based formulations can overcome the limitations of the previously licensed unconjugated Vi capsular polysaccharide vaccines, which suffer from poor immunogenicity in young children and require repeated doses.[132]

5.2.2. Glycoconjugate vaccines against iNTS

Being the main surface-associated polysaccharide in NTS, the OAg has been identified as a possible candidate for the development of iNTS glycoconjugate vaccines. Preclinical evidence has shown that OAg is poorly immunogenic and does not elicit immunological memory if administered alone,[145] owing to its polysaccharide nature (usually associated with a T-independent immune response). However, studies in animal models have revealed that conjugation of *Salmonella* OAg with carrier proteins induces bactericidal antibodies that confer protection against invasive infections.[145–147]

5.2.2.1. iNTS OAg plus CRM197 (iNTS OAg-CRM197) conjugate vaccines. Rondini and colleagues[148] showed that conjugation of *S.* Typhimurium D23580 OAg with CRM197 (OAg-CRM197) was able to induce a protective antibody response in mice and to reduce bacterial load in systemic sites. Among the different D23580 OAg-CRM197 candidates tested, the greatest immunogenicity was associated with OAg populations with the highest acetylation and glycosylation levels along with low or mixed molecular weight.[148] Since the composition of the D23580 OAg is influenced by the *gtr* operon,[42] the expression of which is not controlled by phase variation in D23580, it is unlikely that this strain can escape the immune surveillance provided by OAg-specific antibodies.[42] This further supports the use of D23580 OAg as an iNTS vaccine component, and justifies additional research into other surface-exposed non-phase-variable targets.

Antibodies elicited by the D23580 OAg-CRM197 conjugates have been seen to inhibit the growth of both invasive and noninvasive *S.* Typhimurium strains at very low concentrations; however, they do not offer cross-protection against *S.* Enteriditis.[148] The serovar-specific response has been attributed to the presence of additional O-acetyl groups on the rhamnose of the D23580 OAg, and it has been suggested that partially acetylated O-antigens could cover a wider range of OAg specificities.[149] However, conjugate vaccines against a single serovar have never been intended to provide broad coverage. Given the co-endemicity of *S.* Typhimurium and *S.* Enteriditis in sSA, a bivalent formulation that includes both serogroups would be a more suitable strategy for the development of iNTS glycoconjugate vaccines.[150]

OAg levels of invasive *S.* Typhimurium isolates collected in Kenya have proved to correlate with increased resistance to human serum, whereas no similar association has been observed in invasive *S.* Enteriditis. This latter displays less susceptibility to antibody-mediated killing than *S.* Typhimurium,[43] suggesting that immune mechanisms other than OAg-antibody interaction (such as the antibody-dependent oxidative burst mediated by phagocytes[121]) may be more important in protection against *S.* Enteriditis in sSA.

Importantly, the dissimilar resistance to serum killing observed in the two serovars may have an impact on vaccine efficacy.[149,151] Introducing proper adjuvants, such as aluminum hydroxide (AlOH),[150] cytosine-phosphorothioate-guanine oligodeoxynucleotide[150] or liposomes[152] into the vaccine formulation can boost the immune response against *Salmonella*, potentially offering broader protection. This is well exemplified by a recent immunogenicity study in mice, which showed that Typbar-TCV formulated with AlOH elicited significantly higher anti-Vi IgG titers and greater IL4 and IFN-γ expression than the unadjuvanted version.[153]

5.2.2.2. iNTS Core O-Polysaccharides plus FliC (iNTS COPS:FliC) conjugate vaccines. The administration of *Salmonella* O-polysaccharide linked with proteins of the homologous strain, such as the phase 1 flagellin FliC, is an attractive alternative to conjugation with exogenous carriers. There are several reasons for using FliC as a carrier in iNTS vaccines. First, this enables carrier-induced epitopic suppression to be avoided. Second, anti-flagellin antibodies have proved to be protective against invasive African iNTS in animal models.[134,154,155] Additionally, as FliC is a phase 1 flagellar protein, its inclusion in the vaccine preparation might allow coverage of uncommon monophasic African variants, such as the invasive *S.* Typhimurium I:4,[5],12:i:-, which does not express phase 2 flagella.[156]

S. Typhimurium and *S.* Enteriditis Core O-PolySaccharides (COPS) coupled with FliC (COPS:FliC) have shown similar immunogenicity to OAg-CRM197 conjugates in mice.[149,157] Protection against challenge with the invasive Malian blood isolate *S.* Enteriditis R11 was achieved in both infant and adult mice following immunization with 2 doses of *S.* Enteriditis COPS:FliC adjuvanted with monophosphoryl lipid A, thus providing the rationale for a possible evaluation of this formulation in the youngest.[157] Additionally, the passive transfer of *S.* Typhimurium COPS:FliC-induced maternal antibodies to infant mice proved to confer to the offspring nearly complete protection against lethal challenge with the Malian isolate *S.* Typhimurium D65, thereby providing further preclinical evidence that this vaccine may protect against pediatric iNTS disease in sSA.[158]

More recently, a trivalent formulation containing iNTS COPS:FliC and the licensed *S.* Typhi Vi antigen-based conjugate vaccine Typbar-TCV has shown high immunogenicity and efficacy.[159] Immunization of rabbits with the trivalent typhoid-iNTS conjugate formulation elicited high serum IgG titers against all three polysaccharide antigens. Anti-COPS IgG were primarily directed against serogroup-specific O-polysaccharide epitopes. Post-vaccination rabbit sera mediated substantial bactericidal activity (SBA) *in vitro* against the invasive Malian *S.* Typhimurium D65, whereas lower SBA was reported against an invasive Malian prototype of *S.* Enteriditis.[159] This is in line with the findings of previous studies[151] which hypothesized that invasive *S.* Enteriditis isolates were more resistant than invasive *S.* Typhimurium strains to anti-COPS antibodies-induced complement-mediated killing. Nevertheless, the efficacy of the trivalent typhoid-iNTS conjugate vaccine *in vivo* was high against both iNTS serovars, as the passive transfer of antibodies from the post-vaccination

sera of rabbits was able to protect 100% of *S.* Typhimurium D65-infected mice and 88% of those challenged with the *S.* Enteriditis R11 isolate.[159] The trivalent conjugate vaccine is currently under evaluation in a Phase 1 clinical study involving healthy adults in the United States (clinicaltrials.gov identifier: NCT03981952).

5.3. OMV-based vaccines

Outer Membrane Vesicles (OMVs) are blebs spontaneously released by Gram-negative bacteria; they contain Outer Membrane Proteins (OMPs) and other components, including LPS and OAg. These vesicles can present multiple protective antigens and innate signaling molecules (such as Toll-Like Receptors ligands) to the immune system; thus, they are capable of stimulating different branches of the immune response and potentially possess an intrinsic self-adjuvanting activity.[160] OMVs have been used in vaccine development for the prevention of bacterial infections, such as those caused by *Neisseria meningitidis* (*N. meningitidis*) serogroup B[161] or *Shigella* flexneri,[162] and NTS-derived OMVs have recently been tested in animal models, yielding promising immunogenicity and protection data.[163–165]

One of the main problems of both *Salmonella* OMVs and LAVs is the toxicity of LPS. Several strategies, including dephosphorylation of lipid A[166] or mutation of the *wzy* gene encoding the OAg polymerase involved in LPS synthesis[167] can be applied in order to detoxify LPS without compromising immunogenicity. OMVs shed by *S.* Typhimurium strains carrying targeted LPS mutations have proved to be an effective vaccine candidate, with the potential to cover several serovars.[165]

Another problem associated with the purification of OMVs is the residual presence of flagellin, which can induce deleterious over-activation of the innate immune system via an excessive Toll-Like-Receptor-5-mediated pro-inflammatory response[168] and may cause interference with the immune response elicited against other antigens.[163] Immunization with OMVs derived from flagellin-deficient *S.* Typhimurium has proved able to provide protection against homologous and heterologous serovars (*S.* Enteriditis and *S.* Choleraesuis), suggesting that it might also be a suitable means of achieving cross-reactive immunity.[163,164]

5.3.1. Generalized Modules for Membrane Antigens (GMMA) vaccines

A promising approach to the development of a safe and affordable vaccine against iNTS is the use of OMVs in their native conformation (nOMVs) as a vehicle to deliver iNTS OAg. The shedding level of nOMVs is generally too low for them to be used in vaccine production, but deletion of the *tolR* gene in *Shigella*[169] and NTS[170] species or deletion of the *gna33* gene in *N. meningitidis*[171] can substantially increase the nOMV yield during the blebbing process. Additional mutations, such as detoxification of lipid A,[170] are generally introduced into nOMV-producing organisms in order to reduce the toxicity of LPS. nOMVs shed by these genetically modified bacteria are called "GMMA" and have been used in the development of vaccines against shigellosis[169] and meningococcal disease[171] in African countries.

In very recent years, a bivalent *S.* Typhimurium and *S.* Enteritidis GMMA-based formulation has been proposed as a vaccine candidate against iNTS disease in sSA. Preclinical studies showed that *S.* Enteriditis and *S.* Typhimurium GMMA elicited high OAg-specific IgG and bactericidal responses.[172] The immunogenicity of iNTS GMMA proved to be at least comparable to that observed following immunization with OAg-CRM197 glycoconjugates. Significantly higher bactericidal titers were elicited by monovalent *S.* Typhimurium and *S.* Enteriditis GMMA than by the OAg-CRM197 conjugate vaccines. Importantly, the immunization of mice with a bivalent GMMA formulation substantially reduced the *Salmonella* load at systemic sites following iNTS challenge. In contrast to the OAg-CRM197 conjugates, which predominantly elicited IgG1 antibodies, the iNTS GMMA induced a broad IgG antibody response.

Unlike OAg-CRM197 glycoconjugates, in which variable amounts of high-, mixed- and low-molecular weight O-antigens can be present, the GMMA OAg population is mainly composed of highly glycosylated mixed-molecular weight molecules,[170,172] which have been associated with high immunogenicity.[148] Together with the possibility of inducing an immune response against surface components other than the OAg (such as porins),[173] these features may account for the enhanced immunogenicity and efficacy of GMMA vaccines in comparison with the OAg-CRM197 formulations. Schager and colleagues[173] showed that the protection conferred by iNTS GMMA could be achieved even in the absence of OAg, was predominantly B cell-dependent, and was long-standing, thereby further highlighting the potential of GMMA as an iNTS vaccine candidate. The power of this formulation is exemplified by the success of the GMMA-based *Shigella sonnei* vaccine, which is currently in clinical phase 2 and has yielded promising immunogenicity and safety data.[169,174,175]

In contrast to LAVs and glycoconjugates vaccines, which have been authorized for human use against several pathogens, including *S.* Typhi,[132,142] no formulation based on the GMMA technology has yet been licensed.

5.4. OMP- and T3SS-based vaccines

5.4.1. OMP-based vaccines

OMPs are under investigation for the development of vaccines against *Salmonella* species. The high expression of the outer membrane protein (OMP) PgtE[64,65] and its ability to elicit CD4 + T cell responses in mice[176] have recently prompted research into PgtE B- and T-cell epitopes that can be recognized by vaccine-induced immunity.[176–178] However, the suitability of PgtE as a vaccine antigen against NTS infections has yet to be assessed.

Barat and colleagues[176] showed that, despite its undetectable expression, the siderophore receptor IroN, when used as a vaccine component, provided the longest post-challenge survival times within a set of 37 surface-associated antigens, making it a promising candidate against invasive *Salmonella* infections.

Another OMP, the porin OmpD, has been shown to induce a T-independent B cell-mediated antibody response capable of limiting the disease caused by invasive *S.*

Typhimurium.[179] However, Ashton and colleagues[40] recently found that acquisition of the *bla* CTX-M-15 gene (responsible for resistance against ESBLs) caused disruption of the *ompD* locus in the UK-isolated U60 strain belonging to lineage 2 ST313, which probably originated in Kenya. The absence of OmpD in strains such as U60 may hamper the use of these strains as broad-spectrum vaccines against non-typhoidal salmonellosis in sSA.

5.4.2. T3SS-based vaccines

Components of the T3SS are currently under evaluation as an option for the development of vaccines against iNTS disease. Immunization of BALB/c mice with *Salmonella* LAVs strains carrying a fusion construct based on the SPI-2-T3SS effector SseJ has been seen to cause heterologous antigens to translocate into antigen-presenting cells, thereby inducing a potent CD4 + T cell response.[180] Lee and colleagues[181] reported that the SPI-2-T3SS translocon subunit SseB was only modestly protective in C57BL/6 mice, but that co-administration of flagellin markedly improved vaccine efficacy in comparison with immunization with SseB or flagellin alone, possibly owing to flagellin-induced boosting of the SseB-specific CD4 + T cell response. The group of Jneid[182] found that four oral administrations of the SPI-1-T3SS component SipD adjuvanted with cholera toxin induced 72% protection against a lethal dose of *S.* Typhimurium, thus identifying for the first time an SPI-1-T3SS component as a potential candidate for protein-based iNTS vaccines. Kurtz and colleagues[183] demonstrated that immunization with the SPI-2-T3SS peptide SseI was able to reduce mortality upon *S.* Typhimurium SL1344 challenge. Protection was attributed to a substantial SseI-specific CD4 + T cell response, whereas antibody-mediated and CD8 + T cell responses proved to be less marked.[183,184]

Given the rarity of protective T cell epitopes,[185] the importance of CD4 + T cell immunity in the defense against *Salmonella* infections,[186] and the fact that sera from Malawian HIV-infected subjects contain a high proportion of NTS-specific non-bactericidal anti-LPS IgG,[79,80] T cell epitopes such as SseI have been regarded as promising iNTS vaccine candidates. However, the pseudogenization of the *sseI* gene observed in D23580[54] makes it unfeasible to include SseI epitopes as the sole components of iNTS protein-based vaccines for use in sSA.

A summary of the iNTS vaccines that are currently at the most advanced stage of development is presented in Figure 1.

6. Conclusions

iNTS continues to constitute a significant cause of bacterial bloodstream infections in Africa. The management of iNTS infections in resource-limited settings, such as sSA, is hampered by the nonspecificity of the symptoms of iNTS disease, by the frequency of concurrent life-threatening conditions, by a poor healthcare system and by the lack of accurate diagnostic tools. The emergence of MDR iNTS strains, and particularly the ST313 *S.* Typhimurium clone, further complicates this scenario by limiting the use of previously recommended antibiotics. All these reasons make the development of iNTS vaccines for use in African populations a priority for global health policy-makers.

WGS has offered new solutions for monitoring *Salmonella* resistance patterns and has proved useful in pinpointing key strain-specific mutations that can be exploited for vaccine development. Identifying host risk factors associated with an increased likelihood of NTS bacteremia in Africa is also of the utmost importance in vaccine research, as it helps us to understand the modalities of anti-iNTS immunity in sSA, which, owing to the presence of such comorbidities as HIV and malaria, may be different from those observed in the high-income countries. In this context, GWAS studies are gaining growing interest, since they have uncovered complex genetic

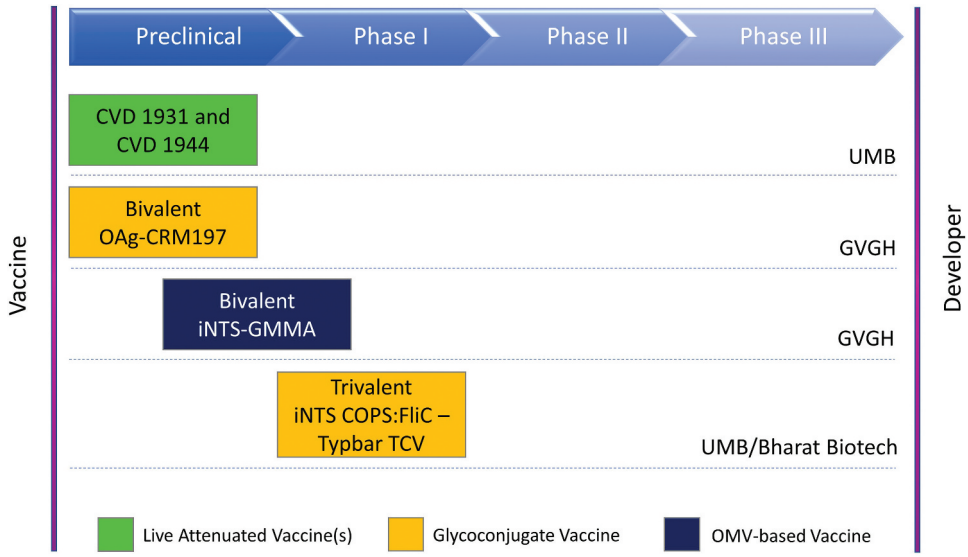

Figure 1. iNTS vaccine candidates currently at the most advanced stage of development. CVD 1931: *S.* Typhimurium D65 *ΔguaBA ΔclpX*;CVD 1944: *S.* Enteritidis R11 *ΔguaBA ΔclpX*;OAg-CRM197: *S.* Typhimurium O:1,4[5],12 OAg conjugated with CRM197 plus *S.* Enteriditis O:1,9,12 OAg conjugated with CRM197;iNTS GMMA: *S.* Typhimurium GMMA containing O:1,4[5],12 OAg plus *S.* Enteridits GMMA containing O:1,9,12 OAg, adsorbed on Alhydrogel.iNTS COPS:FliC – Typbar-TCV: *S.* Typhimurium O:1,4[5],12:H:i plus *S.* Enteriditis O:1,9,12:H:g,m conjugates plus Typbar-TCV vaccine containing *S.* Typhi Vi polysaccharide conjugated with tetanus toxoid.UMB: University of Maryland, Baltimore;GVGH: GlaxoSmithKline (GSK) Vaccines Institute for Global Health.

traits associated with susceptibility to a wide range of conditions, including those which are endemic in Africa, such as malaria,[187] HIV[187] and iNTS disease.[111]

Together with immuno-epidemiological investigations, these studies have shed light on the importance of NTS-specific cell-mediated and humoral immunity in controlling invasive *Salmonella* infections. Recent evidence has shown that antibodies directed against the LPS OAg are associated with a reduced risk of NTS bacteremia in healthy Malawian children.[45,123] However, anti-LPS IgG are present in high concentrations in some HIV-infected individuals, and an excess of these antibodies has shown a lack of complement-mediated *Salmonella* killing *in vitro*,[79,80] highlighting the need for further research into the identification of a serological correlate of iNTS protection.

Given the contribution of the main acquired risk factors (HIV, malaria and malnutrition) to the development of invasive non-typhoidal salmonellosis in sSA, it is likely that public health interventions aimed at reducing these risk factors can lower the burden of iNTS disease in Africa. Indeed, as shown by the successful ART program implemented in 2005 in Blantyre, a decline in iNTS disease can be achieved by reducing the incidence of HIV.[75] Similarly, the strong epidemiological association between malaria and iNTS disease[8] suggests that implementing strategies to control the transmission of malaria parasites (such as the very recent roll-out of the anti-malaria vaccine RTS,S (GlaxoSmithKline) in Malawi, Ghana and Kenya) might yield similar results. Moreover, reducing malnutrition would also contribute enormously to indirectly reducing the incidence of iNTS disease. All these efforts are of the greatest importance, as the iNTS vaccines that are at the most advanced stage of development are currently in the preclinical or early clinical phase, and thus will not be available for human use in the very near future.

The introduction of a trivalent typhoid-iNTS conjugate vaccine (iNTS COPS:FliC conjugates coupled with Typbar-TCV[159]), which is currently in clinical phase 1, constitutes an attractive approach, as this formulation could prevent both typhoid fever and invasive non-typhoidal salmonellosis. Given the high incidence of typhoid fever and iNTS disease in sSA, the use a trivalent formulation capable of covering *S.* Typhi, *S.* Typhimurium, and *S.* Enteritidis may be able to lower the overall burden of invasive *Salmonella* infections in Africa. Moreover, an economically sustainable GMMA-based bivalent vaccine against the African invasive *S.* Typhimurium and *S.* Enteriditis pathovars will enter clinical phase 1 in the coming months. The GMMA method allows high-yield (roughly 100,000,000 doses of vaccine per year even in low-capacity facilities[169]) and low-cost vaccine production, hence constituting an ideal platform for low-income countries.

The introduction of iNTS vaccination in sSA would be accelerated by research to define a clear correlate of protection, the implementation of controlled human challenge models and the possibility to perform large-scale immunogenicity, efficacy and safety trials at multiple African sites afflicted by iNTS. Global research and development funding and broad governmental support are necessary in order to proceed in this direction.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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