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"Driver or passenger" : an integrated epidemiological and experimental perspective on the association between nontyphoidal salmonella infection and colon cancer

Duijster, J.W.

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Chapter 4

REPETITIVE EXPOSURE TO NON-TYPHOIDAL SALMONELLAE IS AN ENVIRONMENTAL RISK FACTOR FOR COLON CANCER

D.M. van Elsland*

J.W. Duijster*

J. Zhang

V. Stévenin

Y.G. Zhang

L. Zha

Y. Xia

E. Franz

J. Sun

L. Mughini-Gras

J.J.C. Neefjes

*equal contributors

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Abstract

During its infectious cycle, *Salmonella* exploits its host by targeting and manipulating essential signalling pathways. This may disrupt cellular integrity and induce oncogenic transformation. Chronic *S. Typhi* infection can indeed cause gallbladder cancer whereas severe non-typhoidal *Salmonella* (NTS) infection is associated with colon cancer (CC). These severe cases, however, represent only a small fraction of all NTS infections occurring in the population. To assess the overall impact of NTS infections on CC development, we performed a retrospective serological study on NTS exposure in CC patients. We observed significant positive association between the magnitude of exposure to NTS and CC risk. Furthermore, repetitive exposure to low NTS doses were shown to recapitulate the tumorigenic effect of a high NTS exposure *in vivo* and repetitive infection with NTS was shown to induce an increase in malignant transformation in predisposed fibroblast cells.

Statement of significance

We observed a higher NTS seroincidence among prospective CC patients and an oncogenic role of repetitive NTS infections in tissue culture and mouse models. As people acquire numerous NTS infections throughout their life these findings raise the importance to consider NTS as an environmental risk factor for CC.

Introduction

Salmonella enterica subspecies *enterica* is a Gram-negative bacterium including more than 2,500 different serovars that can cause gastrointestinal disease and occasionally invasive infection of variable severity. These serovars are commonly divided into two groups. The typhoidal serovars (i.e., Typhi and Paratyphi) are human restricted pathogens that can cause the severe systemic illnesses; typhoid or paratyphoid fever. The non-typhoidal *Salmonella* (NTS) serovars, of which Enteritidis and Typhimurium are among the most common ones in clinical patients, can colonize asymptotically a broad range of animals and usually cause gastroenteritis in humans. As *S. Typhi* and Paratyphi are mainly transmitted between humans via the fecal-oral route, the vast majority of (para)typhoid fever cases occur in densely populated areas lacking access to improved sanitation [1]. Conversely, NTS infections occur worldwide, are common in developed countries and are transmitted mostly from animals to humans via food, as well as directly via animal contact or indirectly via the environment [2,3].

Both typhoidal and non-typhoidal serovars have been linked to human cancer. Globally, the incidence of typhoid fever and gallbladder cancer (GBC) show substantial geographical overlap [4, 5]. This link is further supported by histological findings of *S. Typhi* in tumors of GBC patients from geographic areas with high GBC prevalence [6]. Similar to *S. Typhi*, severe NTS infection is epidemiologically associated with increased colon cancer (CC) risk [7]. Indeed, in a large registry-based nationwide cohort study in the Netherlands, the risk of proximal CC was twice as high among people with a laboratory-diagnosed NTS infection as in the general population [7].

During host cell invasion, *Salmonella* injects over 30 different effector proteins into its host to increase its uptake, intracellular survival and egress [8] (LaRock et al., 2015). Among these effectors, *AvrA*, *SopE*, *SopE2*, *SopB* and *SptP* are known to mediate activation of the hosts β -catenin, MAPK and AKT signalling pathways, respectively [6, 9-12]. The activation of these pathways by *Salmonella* results in transformation of both *in vitro* and *in vivo* models harbouring pre-transformed genotypes, such as partial (heterozygote) or total (homozygote) deficiency of the tumor suppressor genes *Apc* or *Arf*, respectively, and constitutive expression of the protooncogene *c-MYC* [6]. *Salmonella* infection thus triggers the activation of oncogenic pathways and as such contributes to one or more steps in the multi-step oncogenic transformation of pre-transformed cells [13, 14].

The severity of a *Salmonella* infection is determined by (a) host factors, (b) the *Salmonella* virulence profile, and (c) the number of *Salmonellae* ingested [15]. While about 90,000 human salmonellosis cases are reported to public health authorities in Europe each year

[16], this number is based on only those cases needing medical attention, laboratory diagnosis and reporting to public health authorities. It has been estimated that, on average, for every reported salmonellosis case in Europe, approximately 57 *Salmonella* infections go unreported [16]. The reported cases therefore represent mostly severe infections, i.e. a small fraction of all *Salmonella* infections occurring in the population. This has been further supported by serological studies where the rate of the immune response-eliciting exposures to NTS was measured, showing that such exposure vastly exceeds the incidence of clinically overt salmonellosis, with people acquiring numerous mild NTS infections throughout their life [17-19].

As severe or long-lasting *Salmonella* infections may promote colon carcinogenesis by virtue of their higher chance of affecting pre-transformed cells [20], it needs to be understood whether repetitive exposure to NTS also contributes to the multistep CC formation process. To test this, we integrated a serological approach with both *in vivo* and *in vitro* analyses and show that the magnitude of exposure to NTS is epidemiologically associated to CC formation and that *in vivo* exposure to repetitive low doses of NTS contributes to CC in a similar manner as a single high NTS dose. We furthermore report that repetitive NTS infections significantly increase the proliferation of transformed cells in tissue culture experiments. As exposure to NTS is difficult to avoid, these results indicate that *Salmonella* should be considered an environmental risk factor for CC development.

Results

Increased Salmonella seroincidence is associated with increased CC risk

Previously we showed that notified *Salmonella* infections are epidemiologically associated with increased CC risk [7]. However, this study included only reported *Salmonella* infections, which represent a small fraction of the NTS infections that people can acquire throughout life [17-19]. To assess the risk of CC development as a function of the magnitude of NTS exposure, regardless of disease severity, we performed a retrospective matched cohort study on two linked data sets. The first data set was derived from a nationwide cross-sectional serological survey conducted in the Netherlands between October 1995 and December 1996, the so-called 'PIENTER-1' study (De Melker and Conyn-van Spaendonck, 1998). This study established a large serum bank with accompanying epidemiological data representative of the Dutch general population, primarily aimed at immunosurveillance to evaluate the national immunization program. The second data set, obtained from the Netherlands Cancer Registry (NCR), covers all Dutch residents and includes data on patients

diagnosed with CC in the proximal part of the colon since 1998 (ICD-10 codes: C180-C185). We focused our analyses on these colon subsites, as our previous study highlighted a significantly increased risk of cancer after NTS infection only in the proximal but not in the distal part of the colon [7].

By linking the PIENTER-1 study data to CC diagnoses in the NCR data, 36 participants in the PIENTER-1 study were found to have a diagnosed cancer in the proximal colon in the period between January 1st, 1998 and December 31st, 2017 (end of the present study period). Each of the 36 CC patients was then demographically matched with two other PIENTER-1 study participants who were not diagnosed with CC (and were still alive) during the study period (Figure 1A). The characteristics of the total cohort are shown in Supplementary Table 1. The cohort comprised 108 participants (36 CC cases and 72 CC-free individuals), consisting of 42% men and 58% women, with a median age of 63 years (mean 60 years, interquartile range [IQR] 52-68 years) at serum sampling within the PIENTER-1 study.

The serum samples of the 36 CC patients (i.e. 'case') and the 72 persons without CC (i.e. 'controls') were retrieved from the PIENTER-1 serum bank and tested for anti-*Salmonella* IgA, IgM and IgG concentrations using a validated mixed ELISA based on commercially available lipopolysaccharides of the two most common serovars, *S. Enteritidis* (O-antigens 1,9,12) and *S. Typhimurium* (O-antigens 4,5,12). These were used as capture antigens in solid phase and have been extensively validated as a means to determine the rate of infection [21]. For each sample, the concentrations of each Ig isotype were measured and expressed as optical density (OD) units (Supplementary Figure 1). These OD values were then used to estimate the seroincidence of NTS infection, i.e. the average number of NTS infections per person-year, as a measure of NTS infection pressure or force of infection in the person in question. This was done considering the established kinetics of anti-*Salmonella* IgG, IgM, and IgA serum antibody levels following NTS infection [17-19], using an established Bayesian back-calculation model available as an R package called 'seroincidence' [17-19] (Figure 1B). The overall seroincidence was found to be 0.80 (95%CI 0.62-0.98) NTS infections per person-year. When stratified by CC status, the mean seroincidence was 0.94 (95%CI 0.55-1.32) NTS infections per person-year among those who later developed CC, which is higher than the seroincidence of 0.73 (95%CI 0.57-0.89) NTS infections per person-year in the control group. This difference was, however, not statistically significant (HR 1.24, 95%CI 0.82-1.88, $p=0.302$) (Figure 1C, Overall; Supplementary Table 2).

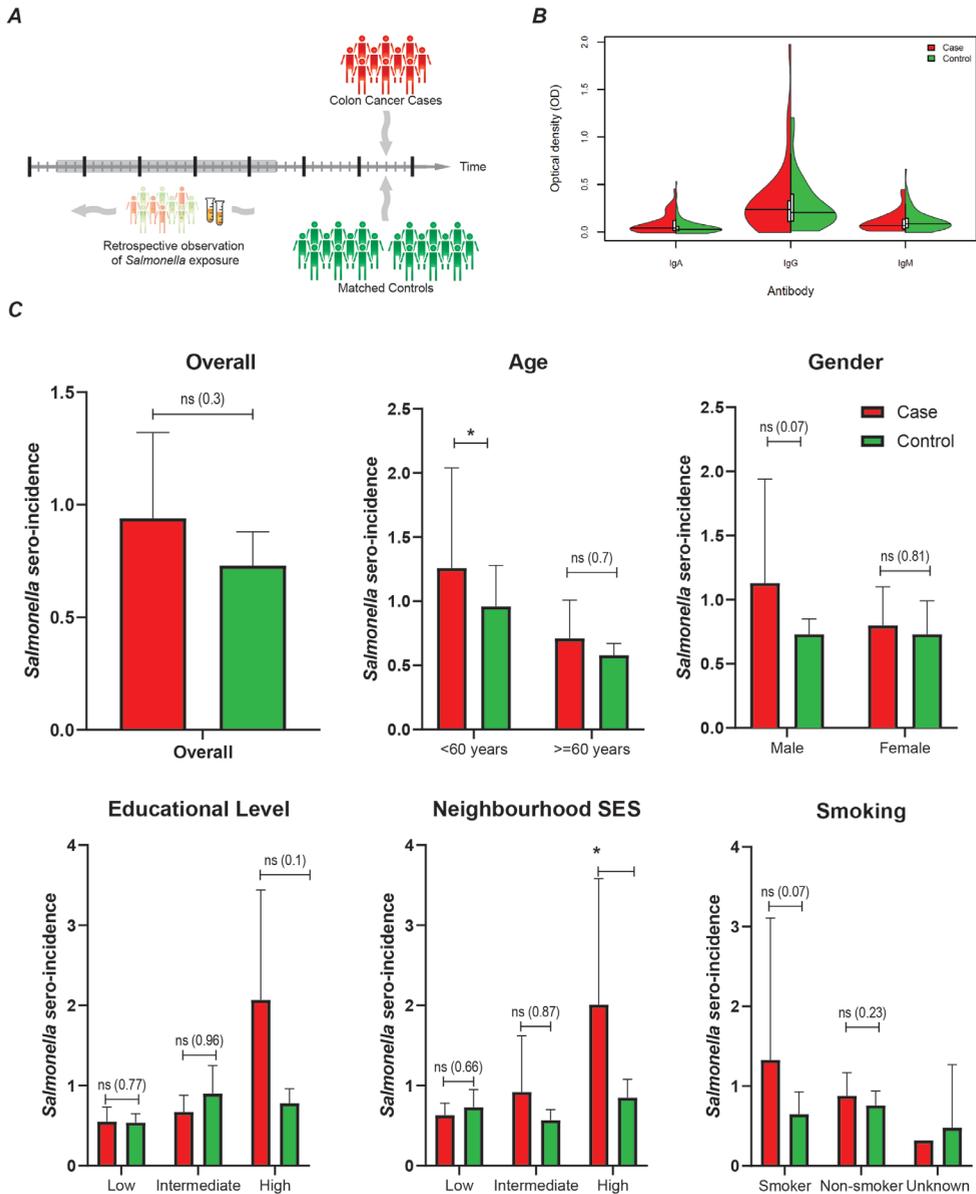


Figure 1. Increased sero-incidence rates among individuals <60 years of age at serum sampling is significantly associated with increased CC risk.

(A) Schematic overview of study design: 36 participants in the PIENTER-1 study with a diagnosed cancer in the proximal colon were demographically matched at a 1:2 ratio with other PIENTER-1 study participants who were not diagnosed with CC (i.e. ‘controls’). The serum samples were tested for anti-*Salmonella* IgA, IgM and IgG concentrations (B) Concentrations of IgA, IgG and IgM anti-*Salmonella* antibodies in cases and controls, expressed in optical density (OD) values. (C) *Salmonella*

sero-incidence rates and colon cancer risk stratified by gender, age, ethnicity, educational level, socioeconomic status and smoking. ns= not significant; the value between brackets shows the p-value of the corresponding hazard ratio. * p-value <0.05.

In our previous study, the increased CC risk concerned specifically individuals with age <60 years at reported NTS infection, as CC risk increases dramatically with age due to a multitude other factors that may dilute the relatively smaller contribution of NTS infection [7]. We therefore stratified the present analysis by age at serum sampling and found that increased seroincidence among individuals <60 years of age at serum sampling was significantly associated with increased CC risk (HR 1.41, 95%CI 1.03-1.94, p=0.033) (Figure 1C, Age; Supplementary Table 2). Other factors like gender, educational level or smoking were not significantly associated with increased seroincidence and CC risk (Figure 1C, *Gender, Educational level, Smoking*; Supplementary Table 2). The only other factor modifying significantly the effect of increased seroincidence on CC risk was living in neighborhoods of high socioeconomic status (SES) (HR 1.32, 95%CI 1.03-1.69, p=0.027), suggesting that the link between NTS seroincidence and CC risk can be enhanced by additional environmental settings (Figure 1C, *Neighborhood SES*; Supplementary Table 2). In conclusion, the serological analyses indicated that a high NTS infection pressure (as defined by seroincidence) in age groups in which age itself can be expected to exert lower oncogenic effects than later in life, may act as risk factor for proximal CC.

Impact of repetitive low dose NTS infections on CC formation in mice

To evaluate whether repetitive NTS infections are capable to trigger cell transformation *in vivo*, a mouse study was designed to compare to role in CC formation of repetitive mild infections versus a single severe infection. Since the severity of a NTS infection is determined by the NTS genotype, host factors and the ingested dose [15], we selected the optimal NTS strain for this long-term CC mice-study through an *in vivo* mortality and morbidity screen of several human clinical NTS isolates (Supplementary Figure 2). Higher NTS doses are reported to give higher attack rates and more severe disease [15]. Mild infections were thus mimicked by infecting mice with a low NTS inoculum of 10 bacteria, whereas a severe infection was mimicked by infecting mice with a high inoculum of 10.000 bacteria; a known and well established dose of Salmonellae for mice studies [22, 23].

Mouse experiments were performed using specific pathogen-free female C57BL/6 mice in a carcinogen azoxymethane (AOM)+ inflammatory agent dextran sodium sulphate (DSS) CC model, that has been extensively used as a model system to investigate the accelerating

effect of NTS infections on the multi-step CC formation process [11]. Single high dose exposures ("Single High Sal.") were performed with single subjection to 10,000 bacteria (equivalent CFU) in a 100- μ l HBSS suspension. Repetitive low dose exposures ("Multiple Low Sal.") were performed with 3 submissions to 10 bacteria (equivalent CFU) in a 100- μ l HBSS suspension. In case of repetitive infections, there was two 4-week delays between exposures (Weeks 1, 4, and 8). As control, non-infected untreated mice were used, as well as non-infected AOM+DSS treated mice (Figure 2A).

Throughout the experiment, changes in body weight were monitored for all groups (Figure 2B). From week 0 to 16, overall weight increased for all these four groups. The increased rates were, however, markedly different between the untreated control group and the AOM+DSS and AOM+DSS-*Salmonella*-infected treatment groups. However, amongst the three treatment groups, no significant differences were observed. In the first 2 weeks after treatment initiation, the increasing rates of mice's body weight from the three treatment groups all slowed down. Until week 2 or 3, the average weight of all three treatment groups were significantly lower than the control group (Figure 2B). As no additional effects on body weight could be observed for any of the NTS exposures, we concluded that the observed body weight changes were solely the result of AOM+DSS treatment

As anticipated from previous studies, AOM+DSS treatment was dominant for tumor formation [11]. Colonic tumors were found to be formed at a similar incidence throughout all treatment groups with no significant differences in case of AOM+DSS-only treatment (tumor incidence: 76.7%; mice with tumor/total mice: 23/30), or in case of both multiple low (71.0%, 22/31) or single high (62.1%, 18/29) *Salmonella* exposures. No tumors were formed in the control group (0/10) (Figure 2C). Yet, tumor sizes of the mice in the groups exposed to multiple low ($p < 0.001$) or single high ($p < 0.01$) doses of NTS were significantly increased compared to the AOM+DSS treated group (Figure 2C and 2D). NTS infection thus appear to accelerate cancer growth in this model and multiple low doses of NTS trigger a similar tumorigenic impact on CC formation as a singular high dose of NTS. Moreover, the location of the colonic tumors was distributed from distal to proximal colon in both multiple low ($p < 0.05$) and single high ($p < 0.05$) NTS exposed groups compared with the AOM+DSS treated group where the tumors were restricted to the distal colon (Figure 2E) [11].

To further assess this attributing effect, colon tissues were subjected to Hematoxylin and Eosin (H&E) staining and pathological analysis (Figure 2I). Lesions of colon tissues of AOM+DSS treated mice revealed low grade dysplasia in the formed tumors. In comparison, both the multiple low dose and single high dose NTS-exposed mice tissues showed

high grade dysplasia and signs of invasive carcinoma. Control mice did not show any abnormalities (Figure 2I). We then evaluated tumor cells and their adjacent tissue growth by BrdU labelling. BrdU labelling was significantly higher in the tumors from both NTS infected groups compared to the AOM+DSS control group ($p < 0.01$), with no significant difference between the single high dose and multiple low dose NTS exposed groups (Figure 2F/G). In the tissue adjacent to tumor, BrdU positive cells in the colon crypts were significantly higher for all treatment groups compared to the non-treated control group ($p < 0.01$). Furthermore, both the low and high dose NTS exposed mice displayed significantly higher BrdU signals in colon crypts than the AOM+DSS control group ($p < 0.01$) (Figure 2F/H). Similar to the tumor tissue, no significant difference in BrdU intensity between the singular and repetitive NTS exposed mice were observed in the colon crypt tissue (Figure 2F/H). These data suggest that both repeated low dose NTS infections or a single high dose NTS infection accelerate proliferation of tumor and tumor adjacent tissue. Colon tissues of mice exposed to both low dose NTS infections and a single high dose NTS infection were found to be colonized by NTS (Figure 2J). Tumor tissues were however colonized with significantly more bacteria in comparison to adjacent non-tumor tissues (Figure 2K), indicating that in case of both low and high inoculates, NTS preferentially accumulated in tumor-tissues.

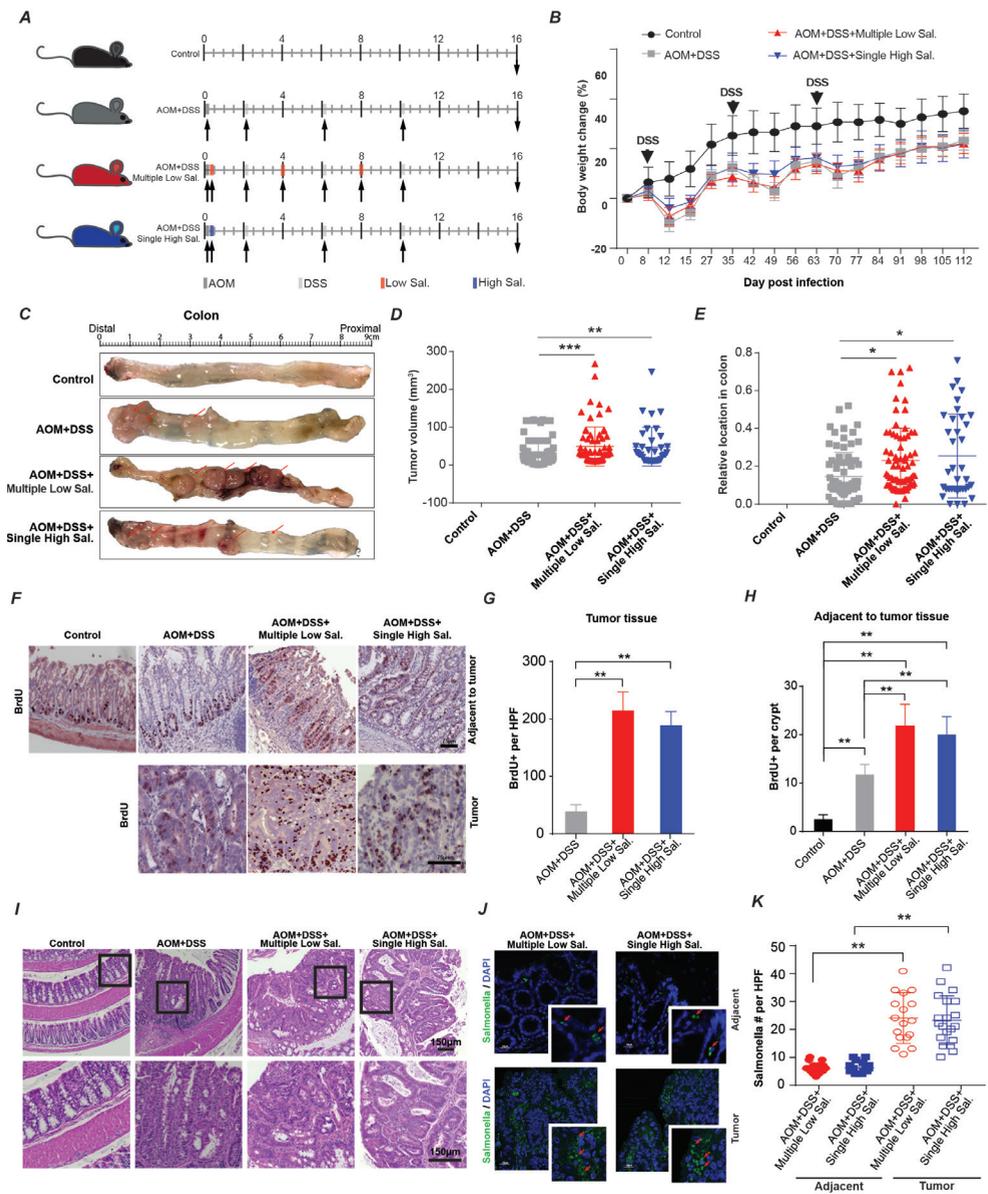


Figure 2. Repetitive Salmonella exposures have tumorigenic impact on colon cancer formation in vivo

(A) Treatment timeline of mice cohort. Mice were infected with either 10 CFU (100- μ l suspension in HBSS) for the repetitive low dose of *S. typhimurium*, with 10,000 CFU (100- μ l suspension in HBSS) for one single high dose, or treated with sterile HBSS (control and AOM+DSS groups) by oral gavage at day 1. The carcinogen AOM was administrated through intraperitoneal injection at day 2 for all groups, except for the control group. After 7-day recovery period, the inflammatory agent dextran

sodium sulphate (DSS) was administered at 2% in drinking water for seven days for all groups except for the control. This DSS treatment was repeated at 5 and 9 weeks. In case of repetitive low-dose infections, oral gavage with 10 CFU (100- μ l suspension in HBSS) of *S. typhimurium* was repeated at 4 and 8 weeks (Figure 2A). The experiment was evaluated at 16 weeks post infection. n=10, 30, 31, and 29 for control, AOM+DSS, AOM+DSS+Multiple Low Sal. and AOM+DSS+Single High Sal. group, respectively. **(B)** Percent of body weight change throughout the experiment for indicated groups of the mice cohort. Data was expressed as mean \pm SD. **(C)** Colonic tumors in situ. Representative colons of indicated groups of the mice cohort at 16 weeks post infection were illustrated. Tumors are indicated by red arrows. **(D)** The tumor volume of indicated groups of within the mice cohort. The data was expressed as mean \pm SD; one-way ANOVA, **p<0.01, ***p<0.001. **(E)** The tumor distribution of indicated groups within the mice cohort. The data was expressed as mean \pm SD; one-way ANOVA, *p<0.05. **(F)** Immunohistochemistry staining of BrdU in normal colon and colonic tumors of the mice cohort. Scale bars 75 μ m. **(G)** Quantification of BrdU staining in tumors from the indicated groups in the mice cohort. The data was expressed as mean \pm SD; one-way ANOVA, **p<0.01, n=6 per group. **(H)** Quantification of BrdU staining in normal tissue from the indicated groups in the mice cohort. The data was expressed mean \pm SD; one-way ANOVA, **p<0.01, n=6 per group. **(I)** Representative images of normal control colon tissue and colon tumor tissues of indicated groups of the mice cohort. *Control* tissue section from different parts of the colon of control mice. Representative section of a tumor in the colon of *AOM+DSS* mice shows a mucosal lesion with low grade dysplasia showing minor gland distortion and nuclear pseudostratification without significant atypia. Some normal colon tissue is still visible on the edge of the lesion. Representative sections through tumors of both *the single high and multiple low Salmonella* exposed mice show high grade dysplasia with mayor gland distortion, cribriform growth and intraluminal cell debris. There is more obvious nuclear atypia with pseudostratification and hyperchromasia. Scale bars 150 μ m. **(J)** *Salmonella* invasion in the colon tissue. Localization of *Salmonella* (red arrow) in adjacent normal tissue and colonic tumor tissue was assessed by immunofluorescence staining with *Salmonella*-specific antibody. **(K)** *Salmonella* invasion in the colon tissue. The number of *Salmonella* was counted per High Pure Field (HPF). Data was expressed as mean \pm SD; one-way ANOVA, ***p<0.001. n=5-6 per group.

Repetitive NTS infection accelerates growth of pre-transformed cells

We have established a minimal tissue culture model for monitoring *Salmonella*-induced transformation [6]. This model includes Mouse Embryonic Fibroblasts (MEF) engineered to mimic two steps towards transformation: *Arf* deficiency (resulting in TP53 inactivation) and overexpression of c-MYC (named *Arf*^{-/-} + c-MYC). Both TP53 mutations and c-MYC overexpression was also observed in gallbladder carcinoma from patients with a history of *S. Typhi* infection (Scanu et al., 2015). To test whether repeated exposures to NTS increased the rate of transformation, *Arf*^{-/-} + c-MYC MEFs were firstly infected with *S. Typhimurium* (MOI 5 and 25) and seeded in soft agar. As previously reported [6], the acquired capacity of the cells to grow and form colonies anchorage independently, which is an established hallmark of transformed cells, resulted from NTS-induced transformation (Figure 3A/B; *Arf*^{-/-} + c-MYC, MOI 5, MOI 25). As control, non-infected *Arf*^{-/-} + c-MYC MEFs were included that failed to induce colony formation (Figure 3A/B; *Arf*^{-/-} + c-MYC, non-infected). Several colonies of NTS-infected *Arf*^{-/-} + c-MYC MEFs were then isolated from soft-agar and cultured under normal

2D cell culture conditions. Throughout culturing no remaining NTS was observed in these cells, as reported previously [6]. These procedures resulted in the establishment of *Arf*^{-/-} + c-MYC MEFs cell lines with a history of NTS infection (hereafter referred to as transformed MEFs). Following re-seeding of the transformed cells, a subset of cells remained able to form colonies in soft agar, as reported previously [5] (Figure 3A/B). To evaluate the effect of repeated NTS exposures on cell transformation, transformed MEFs were re-infected prior to re-seeding in soft-agar, which yielded significantly more colonies (Figure 3A/B; transformed, *comparing non-infected with MOI 5 and MOI 25*). This increase was NTS-dose dependent, as an MOI of 25 resulted in significantly more colonies than an MOI of 5 (Figure 3A/B; transformed, *comparing MOI 5 to MOI 25*). Remarkably, colonies of the transformed cells were also larger following a re-infection, indicating that these colonies proliferated faster than the reseeded *Arf*^{-/-} + c-MYC MEFs or non-infected transformed MEFs (Figure 3C). Increased transformation upon repeated infections was found to be consistent amongst various subsets of the NTS-transformed *Arf*^{-/-} + c-MYC MEFs-isolates (Supplementary Figure 3).

NTS preferably infects (pre-)transformed cells in vitro

As observed in our mouse cohort, tumor tissues were significantly more colonized by NTS in comparison to adjacent non-tumor tissues. In line with these observations it has been reported that NTS preferentially accumulates in tumors when compared to other organs a week after systemic injection [24]. Moreover, specific targeting of host cells by NTS has been reported for particular morphological and microenvironmental features [25]. To evaluate whether NTS specifically targets (pre-)transformed host cells, we compared NTS infection efficiency of MEF cell lines that harbored either one (*Arf*^{-/-}) or two pre-transforming-mutations (*Arf*^{-/-} and c-MYC) to the NTS infection efficiency of transformed MEFs. Intracellular bacterial counts of transformed MEFs were found to be significantly higher than the intracellular bacterial counts of the pre-transformed *Arf*^{-/-} and *Arf*^{-/-} + c-MYC MEFs, indicating that transformed MEFs are more susceptible to NTS invasion than pre-transformed MEFs (Figure 3D). Moreover, intracellular bacterial counts of *Arf*^{-/-} + c-MYC MEFs were significantly higher than the intracellular bacterial counts of *Arf*^{-/-} MEFs, also correlating infection efficiency to transformation state (Figure 3D). The selectivity of NTS for infecting transformed MEFs was further confirmed in a mixed culture of pre-transformed *Arf*^{-/-} MEFs and transformed MEFs. Increasing the proportion of transformed MEFs within the overall cell population correlated with a similar increase in total intracellular NTS numbers, further illustrating that transformed MEFs are infected by NTS with higher efficiency (Figure 3E). Fluorescence microscopy of NTS infected *Arf*^{-/-} c-MYC MEFs and transformed MEFs confirmed higher numbers of

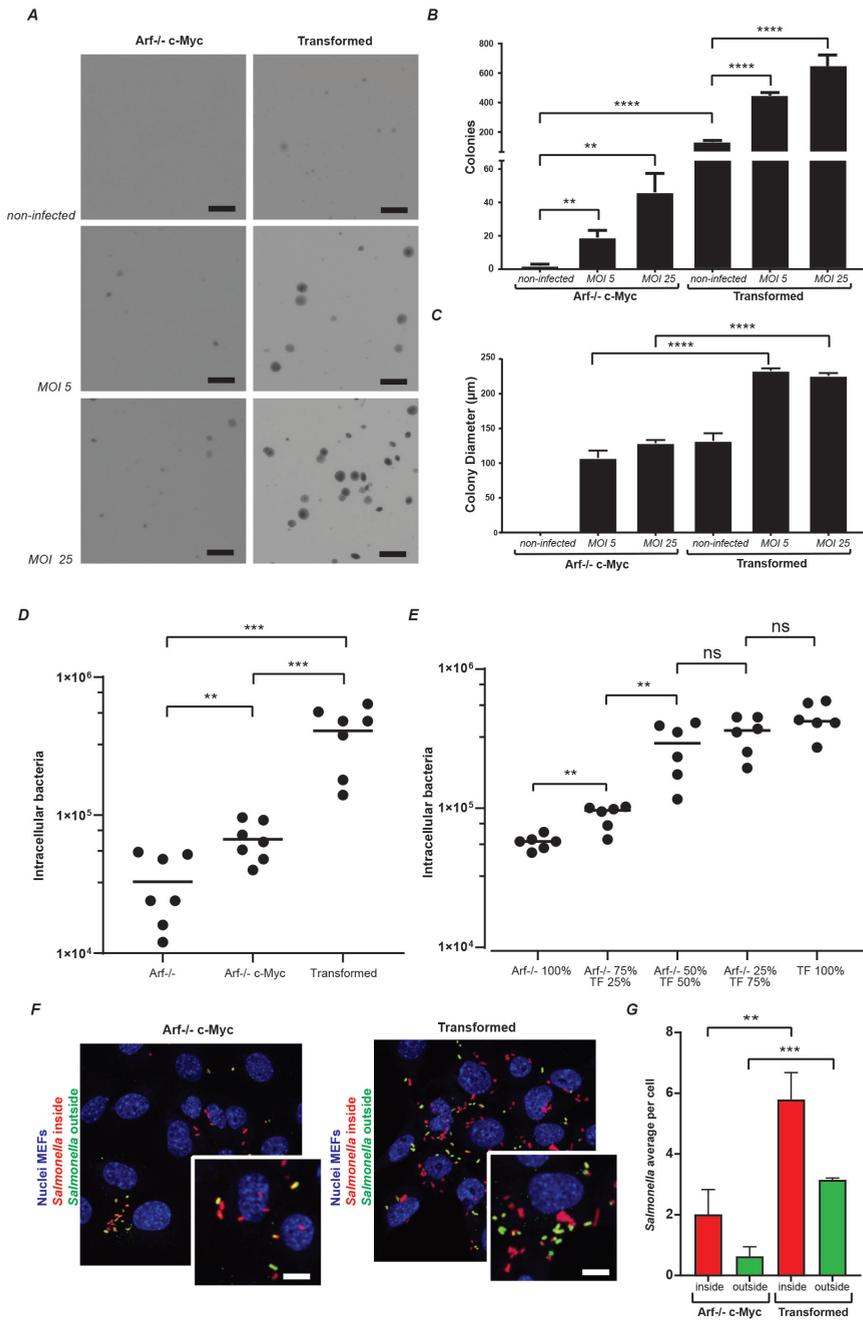


Figure 3: *Salmonella* preferentially infects (pre-)transformed cells, and repetitive *Salmonella* infections increase cellular transformation *in vitro*

(A) Representative images of anchorage-independent growth assay of *Arf*-deficient MEFs overexpressing c-MYC that have not been previously exposed to *Salmonella* (top panels: *Arf*^{-/-} c-MYC), and transformed *Arf*-deficient MEFs overexpressing c-MYC that have been previously exposed to *Salmonella* (bottom panels: *Transformed*). *Arf*^{-/-} c-MYC or transformed MEFs either non-infected, infected with an MOI of 5, or infected with an MOI of 25 are indicated in the left, middle and right panel, respectively. Images represent at least three independent experiments with technical triplicates. Scale bar 750 μ m. **(B)** Average number of soft-agar colonies per well of *Arf*^{-/-} c-MYC and transformed MEFs overexpressing c-MYC that have been either non-infected, infected with an MOI of 5, or infected with an MOI of 25. Results derive from at least three independent experiments with technical triplicates; one-way ANOVA, ** $p < 0.01$, **** $p < 0.0001$. **(C)** Average colony diameter of anchorage-independent growth of naïve and transformed *Arf*-deficient MEFs overexpressing c-MYC that have been either non-infected, infected with an MOI of 5, or infected with an MOI of 25; one-way ANOVA, **** $p < 0.0001$ **(D)** CFU counts of intracellular bacteria in *Arf*^{-/-}, *Arf*^{-/-} c-MYC and transformed MEFs after infection with *Salmonella* at MOI 25 for 1 hour **(E)** CFU counts of intracellular bacteria in mixed populations of *Arf*^{-/-} and transformed MEFs after infection with *Salmonella* at MOI 25 for 1 hour **(F)** Representative images of intra-(inside) and extracellular (outside) *Salmonella* bacteria in *Arf*^{-/-} c-MYC and transformed MEFs after infection with *Salmonella* at MOI 25 for 1 hour. Scale bar 10 μ m. **(G)** Quantification of intra-(inside) and extracellular (outside) *Salmonella* bacteria in *Arf*^{-/-} c-MYC and transformed MEFs after infection with *Salmonella* at MOI 25 for 1 hour; one-way ANOVA, ** $p < 0.01$, *** $p < 0.001$.

Salmonellae in transformed MEFs compared to pre-transformed MEFs. To distinguish intracellular NTS from cell surface bound NTS (i.e., not eliminated during washing steps), we used an NTS strain constitutively expressing a dsRed fluorophore and immunolabelled the extracellular salmonellae. Noteworthy, NTS counts at the cell surface of transformed MEFs were also higher in comparison to pre-transformed MEFs, suggesting that the transformed state increased the host cell-adherence of NTS (Figure 3F/G). Together, our in vitro data demonstrate a privileged tropism of NTS for cells with the highest level of transformation.

Discussion

Environmental factors are important drivers of CC [26], and colonic microbiota play an important role in both the development and progression of CC [27-29]. In addition to microbial factors that directly affect the genomic integrity of epithelial cells, such as genotoxins [30,31], NTS infections have shown to induce oncogenic transformation of pre-transformed cells upon targeting and manipulation of essential signalling pathways [6]. In long-lasting and severe NTS infections, NTS are more likely to encounter a pre-transformed cell increasing the risk of oncogenic transformation. This has already been suggested by the epidemiological association between severe NTS infections and increased CC risk [7]. It then follows that the sum of multiple NTS infections, which people are known to acquire throughout life [17-19], are similarly conceivable to induce CC.

To test this hypothesis, we assessed the risk of CC development as a function of the magnitude of NTS exposure using a retrospective serological study. This provided unique insights into the frequency of NTS infections and revealed an increased risk of developing CC among people with increased NTS seroincidence before 60 years of age, so before advanced age becomes a dominant risk factor in itself. Indeed, after 60 years of age, CC risk increases considerably due to a multitude of factors that would dilute the relatively smaller effect of NTS infection [7].

The magnitude of exposure to NTS was found to be significantly associated with CC risk among people with a high SES. A higher SES is often associated with a more sedentary occupation (i.e. the so called 'white collar' professions), which is a known risk factor for CC [32]. Hence, it seems that colon carcinogenesis fueled by increased exposure to NTS interacts with other drivers of CC. Specifically, depending on the presence or absence of other risk factors for CC like age and lifestyle (which SES is a proxy for), an effect of NTS infection on CC risk is more evident.

Previously high doses of NTS were shown to contribute to CC formation in pre-transformed mice models [6, 11]. Here we observed that multiple low doses of NTS accelerate *in vivo* tumor formation in a similar manner as a single high dose, thereby indicating that repetitive low dose exposures to NTS triggers tissue and tumor proliferation in a comparable manner as a single high dose NTS exposure. Moreover, after both repetitive low dose infections and a single high dose infection, NTS similarly colonized mice's colon tissues at the end of the study, as deduced by pathology. This could be attributed to our observation that (pre-)transformed cells are more efficiently infected by NTS, whereby a repetitive low inoculum could suffice to target and colonize (pre-)transformed tissues with similar efficiency as a high inoculum. NTS persistence at the end of the *in vivo* study suggests acceptance of the infection without sign of inflammation as deduced from the fact that no significant differences in serum cytokines and chemokines were observed between the groups at that point (Supplementary Figure 4). This is in line with previous studies, in which the NTS infection induces high immune cytokines in the blood of the mice after one week, but then drops to the normal level at 10 weeks post infection [33]. These outcomes furthermore demonstrate that NTS infection did not induce immunomodulatory mechanisms in mice. These mechanism thus do not seem to be involved in the here observed NTS-induced CC formation.

The higher NTS seroincidence among prospective CC patients and the oncogenic role of recurrent low dose NTS infections observed in tissue culture and mouse models identify recurrent low dose NTS infections as a cumulative risk factor for CC development. Low dose

NTS infections can be easily obtained from many sources. Indeed, numerous NTS serovars colonize animals and environmental reservoirs, with *S. Enteritidis* transmission being essentially foodborne, whereas *S. Typhimurium* is virtually ubiquitous [32]. While exposure via food can in principle be prevented, elimination of environmental exposure to NTS is practically impossible. Like sunlight, mild and recurrent NTS infections may represent a hitherto unknown environmental risk factor for CC that cannot be avoided and this may be the case for other cancers and bacterial species as well [34].

Methods

Sero-epidemiological study design

A retrospective matched cohort study was performed based on two linked data sets. The first data set derived from a nationwide cross-sectional serological survey conducted in the Netherlands in October 1995-December 1996, the 'PIENTER-1' study [35] (De Melker and Conyn-van Spaendonck, 1998). The design and rationale of PIENTER-1 are described in detail elsewhere [35] (De Melker and Conyn-van Spaendonck, 1998). In brief, a two-stage cluster sampling design, with 48 municipalities nested in five study-defined regions and age-stratified random sampling applied within these municipalities, was performed. In total, 18,217 people were invited to complete an epidemiological questionnaire and to donate a blood sample. Informed consent was obtained for all participants. Data on the neighbourhood socio-economic status (SES: classified as low, intermediate, and high, based on a standardized index including income, occupation, and education) per postal code area was obtained from Statistics Netherlands (www.cbs.nl). In total, 9948 persons provided a serum sample. The second data set, maintained by the Dutch Association of Comprehensive Cancer Centres (IKNL) (www.iknl.nl), was derived from the Netherlands Cancer Registry (NCR). This registry covers all residents in the Netherlands, the data are more than 95% complete, and includes data on patients diagnosed with CC (ICD-O-3 codes: C180-C189) since 1990. These data also include the colon subsite (proximal, distal) in which the tumour has been found.

Statistics Netherlands (CBS) acted as a trusted third party for data anonymization and linkage by adding a Record Identification Number (RIN) as unique identifier for each individual in the two data sets. Birth date, gender, residence location, and date of registration formed the basis for the derivation of the RIN numbers. To this end, CBS used a reference database containing all mutations due to death or relocation in the Dutch population, including a complete housing history of all Dutch residents. After the RIN numbers were added, all

personal identifiers were removed. Based on RIN numbers, the participants of the PIENTER-1 study were linked to the NCR data on patients with diagnosed CC.

All data sets were cleared from duplicates. CC patients with a date of diagnosis falling after the end of the study period (December 31st, 2017) were censored. As a previous study highlighted a significantly increased risk of cancer only in the proximal part of the colon after reported NTS infection [7], we excluded cases with cancer in the distal part of the colon. After linking the PIENTER-1 records to those of the CC patients in the NCR data set, 36 matches were found, i.e. 36 participants in PIENTER-1 who were diagnosed with cancer in the proximal colon in the period between January 1st, 1998 and December 31st, 2017 (end of the present study period). Each of these 36 CC patients was matched at 1:2 ratio to other PIENTER-1 participants who were not diagnosed with any CC and did not die during the study period. Matching was based on age (± 1 year), gender, self-reported educational level (low=primary, lower vocational or lower secondary education; intermediate=intermediate vocational, intermediate secondary or higher secondary education; high=higher vocational and university education), and smoking behavior (smoker, no-smoker, unknown), as reported in the PIENTER-1 study.

Median follow-up time (i.e. time between entry into the cohort and CC diagnosis for the cases or censoring for the matched controls) was 13 years (mean 12 years, IQR 6-16 years), amounting to 1293 person-years at risk in total. The median age at exit from the cohort (i.e. CC diagnosis for the cases or censoring for the matched controls) was 75 years (mean 72 years, IQR 65-80 years). The cohort was mainly composed by persons with a low to intermediate educational level and living predominantly in neighborhoods of low socio-economic status (SES) (Table 1).

Serological analyses and seroincidence calculation

The serum samples of the 36 CC cases and the 72 persons without CC (i.e. 'controls') were retrieved from the PIENTER-1 serum bank and tested for anti-*Salmonella* IgA, IgM, and IgG concentrations using a validated mixed ELISA based on commercially available lipopolysaccharides (SIGMA, Copenhagen) of the two most common serovars, namely Enteritidis (O-antigens 1,9,12) and Typhimurium (O-antigens 4,5,12), as capture antigens in solid phase. A detailed description of this ELISA and its validation has been published previously [21]. For each sample, the concentrations of each Ig isotype were measured separately and expressed as optical density (OD) units. These OD values were then used to estimate, for each sample, the seroincidence of NTS infection, i.e. the average number of NTS infections per person-year as a measure of NTS infection pressure (or force of

infection) in the person in question. This was done using the Bayesian 'back-calculation' model provided for in the R package called 'seroincidence', which has been described in detail elsewhere [17-19] and has been adopted as the standard seroincidence calculator by the European Centre for Disease Control (ECDC) (<https://ecdc.europa.eu/en/publications-data/seroincidence-calculator-tool>). In brief, the model is based on the kinetics of IgG, IgM, and IgA observed during an 18-month follow-up study with repeated bleeding of 302 Danish adult patients with stool culture-confirmed NTS infections. The model used these data as reference values for peak levels and decay rates of IgG, IgM, and IgA concentrations over time after *Salmonella* infection so that the Ig values measured in a sample can be modelled as a function of time since last seroconversion, taking into account inter-individual variation, thereby estimating an annual seroincidence for any observed set of Ig values in a single sample. This model has been used in several studies on immuno-dynamics of NTS [18, 19] and has been adapted to *Campylobacter* [36- 39] and *Yersinia enterocolitica* [40] as well.

Statistical analysis of sero-epidemiological data

The goal of the analysis was to assess whether NTS seroincidence was a significant predictor of CC. Cox proportional hazards models with attained age as the time scale were used to calculate hazard ratios (HR) and 95% confidence intervals (95%CI) for CC (failure event) as a function of the NTS seroincidence (continuous predictor variable). Follow-up started at cohort entry (i.e. serum sampling at the PIENTER-1 study) and ended at CC diagnosis for both the cases and their matched controls (censoring). As the follow-up time was equal for the members of each matched set, the Breslow method for ties in follow-up time produced HRs that corresponded to risk ratios [41]. A clustered sandwich estimator for variance was used to account for the matched sets, which shown to yield robust estimates of variance for hypothesis testing [42] and generally produce results comparable to frailty models [43].

Stratified analyses were performed according to age at sampling (defined as <60 vs. ≥60 years, as this was the mean age in our sample and a previous study showed that the potential effect of NTS infection on CC development is unlikely to be observed after that age given the prominent role of other risk factors that may 'dilute' the effect of the infection) [7], as well as gender, neighbourhood SES, educational level, and smoking status, to assess whether there were modifications of the effect of NTS infection pressure on CC risk according to these strata. The two-way interactions between seroincidence and the aforementioned variables were assessed in separate models adjusted for the other variables. However, to avoid collinearity due to the strong association between educational level and neighbourhood SES, only one of these two factors was included as covariate based on the best model fit

as revealed by the Akaike's information criterion. Proportional hazard assumptions were verified using graphical and residual-based methods and found to be met. Statistical analysis was performed using STATA 15 (StataCorp, LP, College Station, Texas, USA).

Animals and ethics statement

Female and male C57BL/6 aged 6-8 weeks mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). All animal work was approved by University of Illinois at Chicago Committee on Animal Resources (AAC 18-216). Euthanasia was accomplished via sodium pentobarbital (100 mg per kg body weight) I.P., followed by cervical dislocation. All methods were carried out in accordance with the approved guidelines by the Committee on Animal Resources.

Bacterial strains for animal model and growth condition

Six clinical isolates, including *Salmonella* Typhimurium 1090200009, *Salmonella* Enteritidis 1090301578, *Salmonella* Enteritidis 1091100412, *Salmonella* Typhimurium 1090601671, *Salmonella* Typhimurium 1090404321, and *Salmonella* Enteritidis 1091302626, were used for the morbidity and mortality animal studies. The clinical isolate *Salmonella* Typhimurium 1090404321 was used for the long-term colon cancer mouse model study. Non-agitated microaerophilic bacterial cultures were prepared by inoculating 0.01 mL of a stationary-phase culture to 10mL Buffered Peptone Water (Sigma-Aldrich, St. Louis, MO, USA) followed by overnight incubation (~18 hours) at 37°C.

NTS-infected CC mouse model

Mice experiments were performed using a specific pathogen-free male and female C57BL/6 AOM+DSS CC model, that has been extensively used as a model system to investigate the accelerating effect of NTS infections to the multi-step CC formation process [11]. Animal experiments were performed with 50 male and 50 female C57BL/6 mice aging 6-7 weeks old (The Jackson Laboratory, Bar Harbor, ME, USA) [11,12]. After setting-down for one week in the animal facility, the mice were infected with either a) a single high dose of 1×10^4 CFU (100- μ l suspension in HBSS) *S. Typhimurium*, b) repetitive low doses of 1×10^1 CFU (100- μ l suspension in HBSS) *S. Typhimurium* or c) treated with sterile HBSS (control) by oral gavage, as previously described [7, 11]. After NTS gavage, the carcinogen AOM was administrated through intraperitoneal injection with the dose based on body weight (10 mg/kg) [11]. After a 7-day recovery period, the inflammatory agent dextran sodium sulphate (DSS) was administrated at 2% in drinking water for seven days. This DSS treatment was repeated at 5 and 9 weeks. In the group of repetitive low dose infections, oral gavage with

1×10^1 CFU (100- μ l suspension in HBSS) of *S. Typhimurium* was repeated at 4 and 8 weeks. Throughout the experiment, mice were weighed and monitored regularly. At 16 weeks post NTS infection, tumors and tissue samples were collected. Tumor counts and measurements were performed in a blinded fashion under a stereo-dissecting microscope (Nikon SMZ1000, Melville, NY, USA). The tumor volume (V) was calculated with caliper measurements using formulas $V = (W^2 \times L)/2$ as described before [44].

Histological testing

Tissues were fixed in 10% neutral buffered formaldehyde for 4-12 hours, then transferred into 70% ethanol and processed by standard techniques. Sections (4 μ m) were stained with hematoxylin and eosin.

Immunohistochemistry

Tissues were fixed in 10% neutral-buffered formaldehyde for overnight, then transferred into 70% ethanol the next day and processed by standard techniques. Immunohistochemistry staining of target protein was performed on paraffin-embedded sections (4 μ m). Briefly, the paraffin sections were baked in an oven at 56 °C for 30 minutes. The slides were deparaffinized and rehydrated in xylene, followed by graded ethanol washes at room temperature. Antigen retrieval was achieved by boiling the slides in a microwave oven with 0.01 M, pH 6.0 sodium citrate buffer. Then, the slides were incubated in hydrogen peroxide (3% H₂O₂ in PBS) for 10 minutes, followed by incubation in 5% fetal bovine serum/PBS for 1 hour at room temperature. Purified Anti-BrdU antibody (Abcam, Cambridge, MA, USA) was used in this study [45].

Immunofluorescence and Confocal Imaging

Fresh tumors were fixed in 10% neutral buffered formalin followed by paraffin embedding. For immunofluorescence staining, slides were incubated in 5% bovine serum albumin (BSA) with 0.1% goat serum in PBS for 1 hour at room temperature to reduce nonspecific background. The samples were incubated overnight at 4°C with primary antibody at 1:100 dilution. The sections were then incubated with secondary antibodies and DAPI for 1 hour at room temperature, and they were examined with confocal microscope as described before [45, 46]. The mouse monoclonal antibody for *S. Typhimurium* 0-4 (Santa Cruz, Dallas, TX, USA) was used in this study.

Luminex immunoassays

The cytokines and chemokines in the plasma samples from the studied animals were assessed using the ProcartaPlex Mouse Cytokine/Chemokine Convenience Panel 1 26plex

(Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instruction. Briefly, after adding magnetic beads, 25 μ l of plasma samples were added and followed by detection of antibody and streptavidin-PE provided by the kit. The plate was read on a MAGPIX™ system platform (Millipore Sigma, Burlington, MA, USA) after adding reading buffer.

Statistical analysis of mice experiments

For the mouse model related experiments, data were expressed as mean \pm SD. One-way ANOVA was performed to the statistical analysis in the animal studies. All statistical tests were two-sided, and p-values <0.05 were considered statistically significant. The statistical analyses of experimental data were performed with GraphPad Prism 5.

Bacterial strains and cell lines for in vitro experiments

S. Typhimurium strain SL1344 was a courtesy of S. Méresse [47]. Mouse Embryonic Fibroblasts (MEFs) were derived from Arf-deficient C57BL/6 mice. MEFs overexpressing c-MYC were generated by retroviral transduction using a pLZRS-GFP(ires)-HA backbone. MEFs were cultured at 37°C, 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) [6].

In vitro NTS infection, CFU, microscopy and anchorage-independent growth assays

NTS infection of MEFs cells was performed as described previously [9]. In brief, *S. Typhimurium* strain SL1344 was grown overnight at 37°C in LB medium, supplemented with 100 μ g/mL ampicillin throughout the bacterial culturing. The next day, the bacteria were sub-cultured at a dilution of 1:33 in fresh LB medium and incubated for 2 hours at 37°C while shaking. Cells were infected with NTS at the indicated MOI in DMEM medium without antibiotics for 20 minutes at 37°C, 5% CO₂ in a tissue culture chamber and then incubated in the presence of 100 μ g/mL gentamicin (GIBCO) for 1 hour to eliminate extracellular bacteria. In case of CFU or microscopy experiments cells were then lysed and plated on LB plates or fixed with 4% PFA for 10 min at room temperature, respectively. In case of anchorage-independent growth assays MEFs were cultured for another 2 hours in the presence of 10 μ g/mL gentamicin. The infected MEFs were subsequently collected and resuspended in DMEM medium supplemented with 10 μ g/mL gentamicin and 0.35% low melting point agarose (UltraPure™, Invitrogen) and were poured on a soft agar bottom layer consisting of 0.7% low melting point agarose in DMEM with 10 μ g/mL gentamicin. Anchorage-independent cell growth and number of soft agar colonies were assessed after 1-3 weeks of incubation at 37°C, 5% CO₂ using GelCount™ (Oxford Optronix, UK). For microscopy analysis fixed slides were stained with rabbit polyclonal anti-*S. Typhimurium* LPS (Difco, Detroit, MI) and

DAPI (Life Technologies). Images were acquired using a Leica TCS SP8 (Leica Microsystems, Wetzlar, Germany) at 40x or 63x magnification.

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Supplementary tables

Supplementary Table 1. Baseline characteristics of the study cohorts

	Individuals who developed colon cancer during the follow-up period	Individuals who did not develop colon cancer during the follow-up period*
Gender		
Male	15 (42%)	30 (42%)
Female	21 (58%)	42 (58%)
Age at entry		
<60 years	15 (42%)	30 (42%)
≥60 years	21 (58%)	42 (58%)
Educational level at entry**		
Low	14 (39%)	28 (39%)
Intermediate	14 (39%)	28 (39%)
High	8 (22%)	16 (22%)
Neighbourhood SES at entry***		
Low	24 (67%)	49 (68%)
Intermediate	5 (14%)	10 (14%)
High	7 (19%)	13 (18%)
Smoking at entry		
Smoker	6 (17%)	12 (17%)
Non-smoker	29 (80%)	58 (80%)
Unknown	1 (3%)	2 (3%)
Follow-up time		
<5 years	6 (16%)	12 (16%)
5-15 years	15 (42%)	30 (42%)
>5 years	15 (42%)	30 (42%)

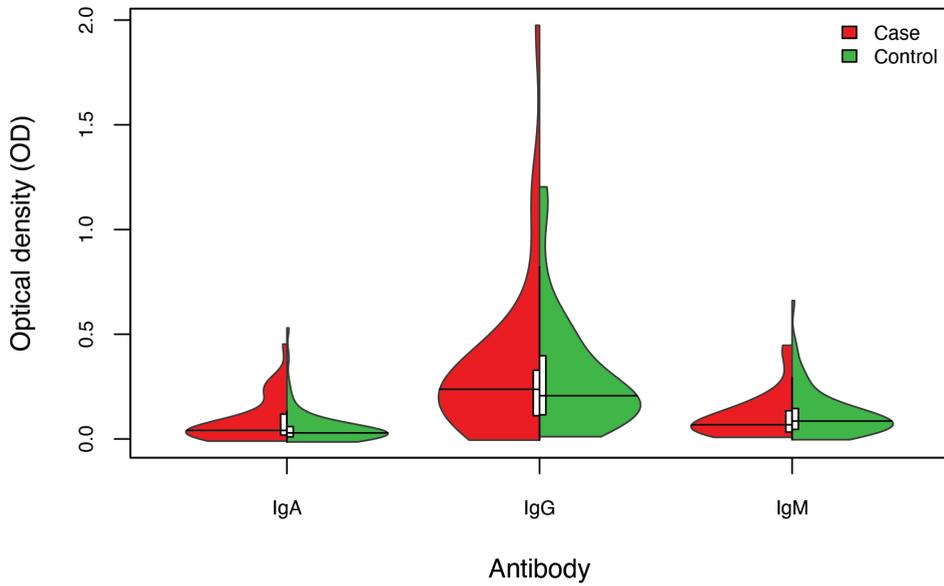
*Matched to the colon cancer patients at a 1:2 ratio based on gender, age at entry (± 1 year), educational level and smoking status. **Low = primary, lower vocational or lower secondary education; intermediate = intermediate vocational intermediate secondary or higher secondary education; high = higher vocational and university education. ***Socio-economic status, classified as low, intermediate and high based on a standard index including income, occupation and education per postal code area ('neighbourhood') obtained from Statistics Netherlands (www.cbs.nl).

Supplementary Table 2. *Salmonella* sero-incidence rates and colon cancer risk by gender, age, ethnicity, educational level, socioeconomic status and smoking.

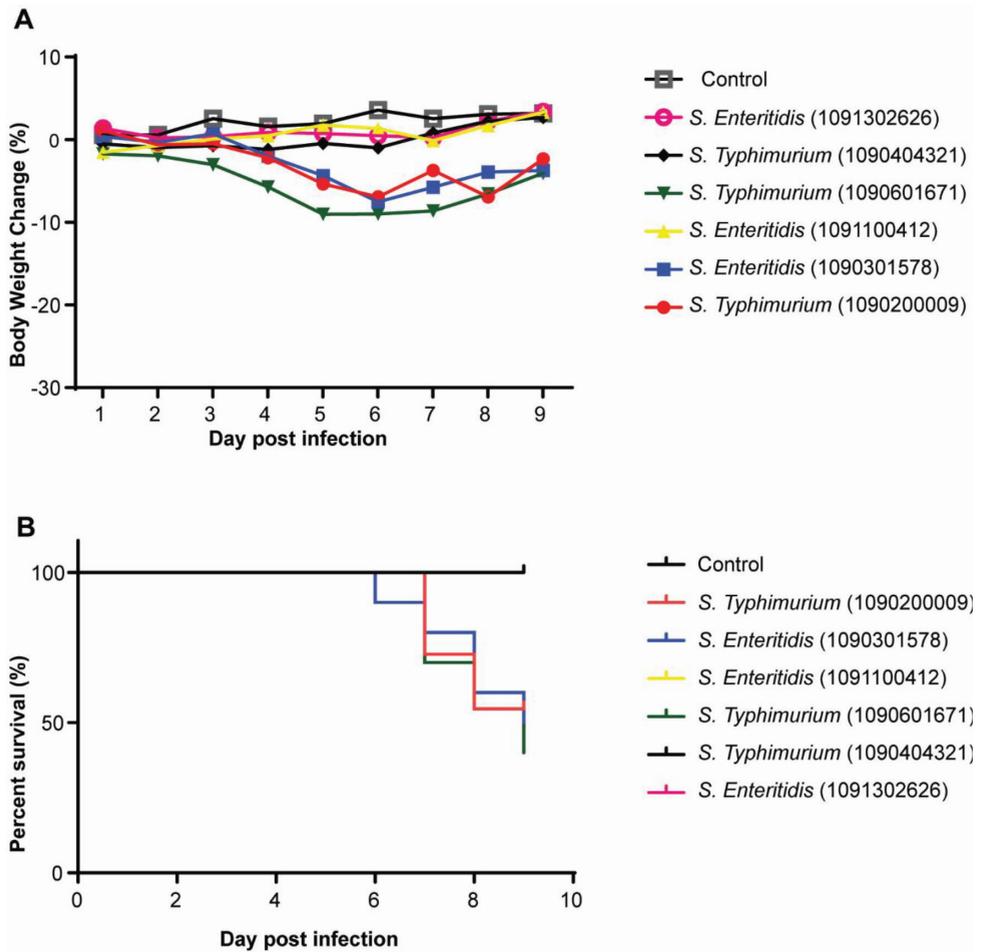
	Mean (95%CI) <i>Salmonella</i> sero-incidence among colon cancer cases	Mean (95%CI) <i>Salmonella</i> sero-incidence among controls	Person-years at risk	Hazard ratio (95%CI)	P-value
Overall	0.94 (0.55-1.32)	0.73 (0.57-0.88)	1293	1.24 (0.82-1.88) [§]	0.302
Age at entry					
<60 years	1.26 (0.48-2.04)	0.96 (0.64-1.28)	617	1.41 (1.03-1.94)[†]	0.033
≥60 years	0.71 (0.40-1.01)	0.58 (0.48-0.67)	676	0.77 (0.29-2.01) [†]	0.704
Gender					
Male	1.13 (0.32-1.94)	0.73 (0.61-0.85)	581	1.46 (0.97-2.22) [§]	0.072
Female	0.80 (0.50-1.10)	0.73 (0.47-0.99)	712	1.06 (0.68-1.64) [§]	0.810
Educational level at entry*					
Low	0.55 (0.38-0.73)	0.54 (0.42-0.65)	424	0.83 (0.23-2.94) [§]	0.771
Intermediate	0.67 (0.47-0.88)	0.90 (0.55-1.25)	564	1.01 (0.59-1.73) [§]	0.965
High	2.07 (0.71-3.44)	0.78 (0.60-0.96)	305	1.26 (0.96-1.66) [§]	0.102
Neighbourhood SES at entry**					
Low	0.63 (0.47-0.78)	0.73 (0.51-0.95)	827	0.87 (0.47-1.60) [†]	0.656
Intermediate	0.92 (0.22-1.62)	0.57 (0.44-0.70)	202	1.03 (0.71-1.49) [†]	0.866
High	2.01 (0.44-3.58)	0.85 (0.65-1.08)	264	1.32 (1.03-1.69)[†]	0.027
Smoking at entry					
Smoker	1.33 (0.00-3.11)	0.65 (0.36-0.93)	242	1.46 (0.97-2.19) [†]	0.068
Non-smoker	0.88 (0.58-1.17)	0.76 (0.57-0.94)	988	1.06 (0.73-1.52) [†]	0.234
Unknown	0.32 (0.32-0.32)	0.48 (0.00-1.27)	63	-	-

*Low=primary, lower vocational or lower secondary education; intermediate=intermediate vocational intermediate secondary or higher secondary education; high=higher vocational and university education. **Socio-economic status, classified as low, intermediate and high based on a standard index including income, occupation and education per postal code area ('neighbourhood') obtained from Statistics Netherlands (www.cbs.nl). §Adjusted for all other variables in the table, except for neighbourhood SES, as it was collinear with the educational level and the inclusion of educational level in the model resulted in a better model fit (lower AIC) as compared to including neighbourhood SES. †Adjusted for all other variables in the table, except for educational level, as it was collinear with the neighbourhood SES and the inclusion of neighbourhood SES in the model resulted in a better model fit (lower AIC) as compared to including for educational level.

Supplementary figures

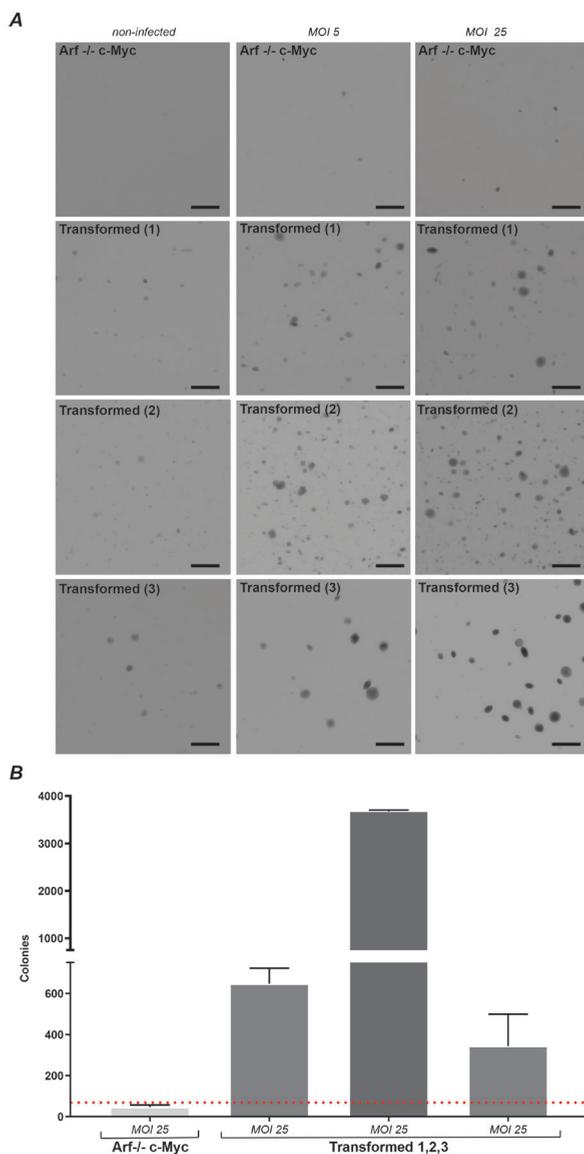


Supplementary Figure 1. Violin plot of the optical density (OD) values of IgA, IgG and IgM of the 36 cases (red) and 72 controls (green).



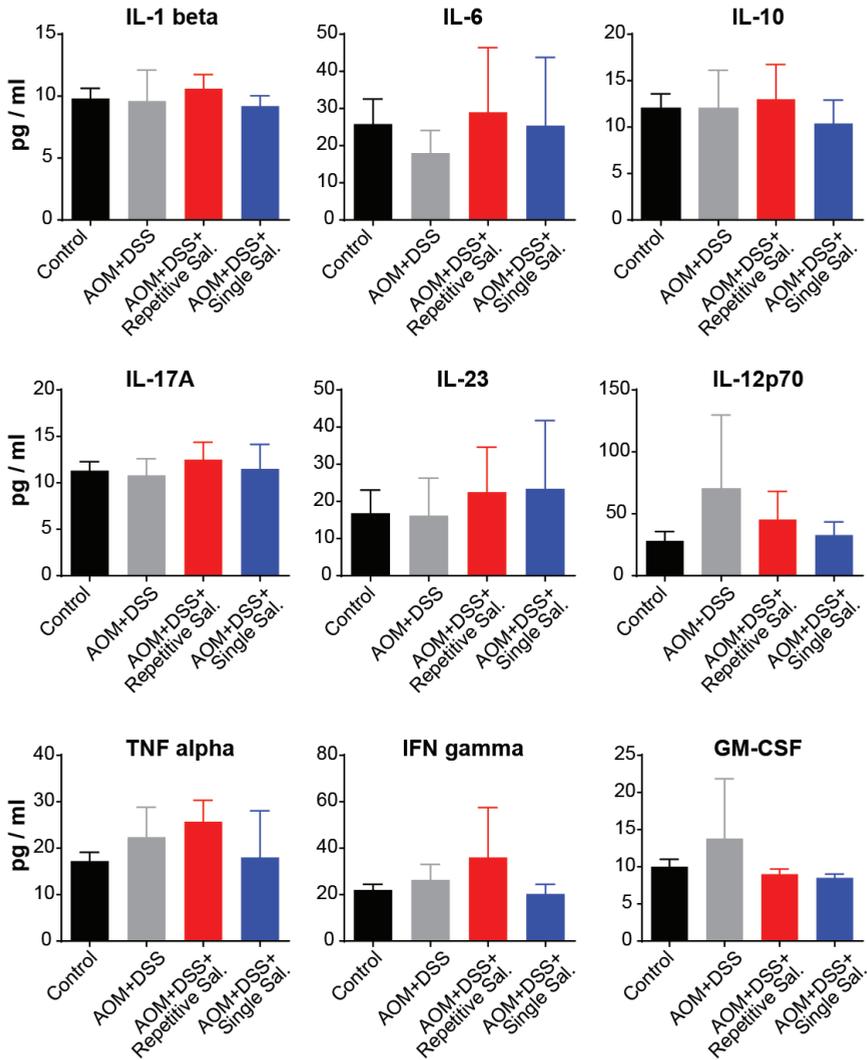
Supplementary Figure 2. Body weight and mortality of the clinical human NTS isolates.

(A) Percent of body weight change throughout the experiment for indicated groups of the mice cohort. The animals (5-female and 5-male per groups) were inoculated with indicated *Salmonella* isolates (1×10^5 bacteria per mouse). Data was expressed by mean \pm SD, $n=10$ mice each group. **(B)** Mortality of the infected animals throughout the experiment for indicated groups. $n=10$ mice each group.



Supplementary Figure 3. Repeated *Salmonella* infection increases cellular transformation.

(A) Representative images of anchorage-independent growth assays of *Arf*^{-/-} c-MYC MEFs that have not previously encountered *Salmonella* (top panels: *Arf*^{-/-} c-MYC) and of 3 *Arf*^{-/-} c-MYC MEFs that did previously encountered *Salmonella* (bottom 3 panels; *Transformed (1)*, *(2)* and *(3)*). *Arf*^{-/-} c-MYC or transformed MEFs either non-infected, infected with an MOI of 5 or infected with an MOI of 25 are indicated in the left, middle and right panel, respectively. Scale bar: 750 μ m. **(B)** Average number of soft agar colonies per well of naive and transformed *Arf*^{-/-} c-MYC MEFs that have been either non-infected, or infected with an MOI of 25. Results are averages of three technical triplicates.



Supplementary Figure 4: Cytokine and chemokines in plasma from mouse model. The cytokines and chemokines in the serum from the experimental animals were evaluated with the Luminex kit as product's instructions. The data was expressed as mean \pm SD; one-way ANOVA, n=5 per group.

