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

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Appendix

SUMMARY

SAMENVATTING

CURRICULUM VITAE

LIST OF PUBLICATIONS

DANKWOORD

Summary

A growing body of scientific literature documents a putative role of commensal and pathogenic bacteria in the initiation and progression of cancers. One such bacterium is nontyphoidal *Salmonella*, which has been associated with colon cancer in a few studies. Yet, a lot is still unknown about the magnitude and underlying mechanisms, including the necessary conditions or 'prerequisites', of the potential colon carcinogenesis promoting effects of *Salmonella*. The main objective of this thesis was to elucidate the role of nontyphoidal *Salmonella* infection in the development of cancers in the gastrointestinal tract, with particular focus on colon cancer. To this end, we performed several complementary analyses based on both experimental and epidemiological study designs.

In **chapter 2, 3 and 4** we investigated whether frequent exposure to zoonotic pathogens such as *Salmonella* is associated with an increased risk of colon cancer. Exposure to *Salmonella* might occur in occupational settings, for instance due to contact with live animals or (raw) products thereof. In **chapter 2** we assessed the association between reported culture-confirmed *Salmonella* and *Campylobacter* infection and occupation in a nationwide registry study in the Netherlands in the period 1999-2016. To this end, we used an internationally agreed upon occupational classification system. Besides analyses of the incidence in occupational divisions within the total dataset, we defined three high-risk groups for zoonotic infection. These high-risk groups include occupations possibly associated with frequent exposure to (low dose) *Salmonella* through 1) contact with live animals and animal manure (e.g., farmers, abattoir workers, veterinarians), 2) processing of foods of animal origin (e.g., cooks and chefs) and 3) sale of products of animal origin (e.g., butchers and cheese store employees). Occupational data was linked to salmonellosis or campylobacteriosis data at the individual person level. We compared the incidence of salmonellosis and campylobacteriosis in the occupational divisions and high-risk groups with the incidence in the matching group (based on *Salmonella* serovar or *Campylobacter* species, calendar year and people's gender and age group) in the total employed population of the Netherlands. The standardized incidence ratios of both salmonellosis and campylobacteriosis were significantly increased in the risk groups involving contact with live animals or animal manure and food sale (1.4-1.8-fold increased). Also, significant excess incidence of reported salmonellosis and campylobacteriosis was observed in healthcare-related occupations and several industrial occupations. In addition to reported infections, we compared the serological incidence (or seroincidence) of *Salmonella* or *Campylobacter* in a subset of the employed population. The seroincidence is defined as the estimated number of (in this case *Salmonella* or *Campylobacter*) infections per person per year, which provides

a less biased method for the estimation of the infection pressure (i.e., force of infection), as it is based on seroconversion (i.e., immune response-eliciting infection), rather than clinical disease alone. Little variation was found in the seroincidences between occupational groups or high-risk groups, hence, the observed differences in incidence of reported infections were only slightly reflected by the infection pressure. In **chapter 3**, we used a comparable study design to explore the incidence of colon cancer among different professions and assessed whether the occupational groups with increased salmonellosis incidence were also those more prone to develop colon cancer. In accordance with previous literature, occupation in itself provided little differences in colon cancer incidence, as relatively minor differences in incidences were observed among occupational divisions. Likewise, no increased risk of colon cancer was observed in the three pre-defined high-risk groups with higher incidences of salmonellosis. Significant higher incidences of colon cancer were found for several industrial divisions, as well as a few non-manual occupational divisions associated with more sedentary tasks. The contribution of the major lifestyle-related risk factors of colon cancer and the heterogeneity of these risk factors among occupational groups might have diluted the relatively minor putative effect of *Salmonella*.

In **chapter 4,** we assessed whether the risk of colon cancer is associated with *Salmonella* exposure earlier in life by using serum samples from a Dutch serosurvey, which were linked to colon cancer diagnosis data. To this end, we compared the *Salmonella* seroincidence of 36 people who developed proximal colon cancer >1 year after participation in the serosurvey (i.e., cases) to the seroincidence of 72 matched individuals without a colon cancer diagnosis (i.e. controls). Matching was done on gender, age, educational level, socioeconomic status and smoking behavior. The seroincidence was significantly higher in cases *versus* controls in the subgroup aged <60 years at time of the serosurvey and the subgroup with a high socioeconomic status. Besides the epidemiological analyses, we also investigated whether repeated exposure with a low dose of *Salmonella* can induce a tumorigenic response under experimental conditions. Mice with a predisposition for colon cancer received either three low-dose infections or one high-dose infection with *S*. Typhimurium. Both infected groups of mice, as well as the control group (without infection), developed colon tumors. However, the tumors of infected mice were larger and showed more often high-grade dysplasia. Yet, repeated low-dose infections with *Salmonella* sufficed to induce a similar tumorigenic effect, as no differences were observed between the mice with multiple low- *versus* single high-dose infections. Also, mouse embryonic fibroblasts (MEFs) with a predisposition for cancer were infected with a high or a low dose *S*. Typhimurium. After this first infection, cellular transformation was observed in both groups of MEFs in a soft agar assay. Reinfection of

these transformed MEFs (i.e. with prior exposure to *Salmonella*) with the same low or high dose led to the formation of more and larger colonies as compared to the first infection. Still, two-fold infection with a low dose was more successful as compared to a single high-dose infection (i.e. more and larger colonies).

In **chapter 5,** we continued with an epidemiological study in a Danish cohort to complement the findings of an earlier Dutch cohort study which found an increased risk after reported (severe) *Salmonella* infection. In the Danish cohort, the incidence of colon cancer was compared for individuals with a reported *Salmonella* infection in the past *versus* individuals without a reported *Salmonella* infection in the period 1994-2015. Again, the salmonellosis surveillance data was linked to colon cancer diagnosis data and demographic data. Cox regression showed no overall increased risk of colon cancer after *Salmonella* infection. However, the risk of proximal colon cancer was significantly increased (1.4-fold) >1 year after infection with serovars other than Enteritidis and Typhimurium (the two serovars which cause over 70% of the infections). Remarkably, an over two-fold increased incidence of colon cancer was found among individuals with a *Salmonella* infection less than one year before the cancer diagnosis. These results differ from the findings of the earlier Dutch cohort study which found an increased risk of proximal colon cancer in people aged <60 when infected, particularly when infected with *S*. Enteritidis.

The association between chronic *S*. Typhi infection and gallbladder carcinoma is well established and earlier research showed that also *S*. Typhimurium is able to induce tumorigenesis in gallbladder organoids. Whether nontyphoidal *Salmonella* is associated with a higher risk of biliary tract (including gallbladder) cancer was addressed in **chapter 6**. By comparing the incidence of biliary tract cancer >1 year after *Salmonella* or *Campylobacter* infection with the incidence of biliary tract cancer in the general Dutch population, we found a tendency towards a higher risk of biliary tract cancer after *Salmonella* but not *Campylobacter* infection. Yet, as biliary tract cancer is rare in the Netherlands, the numbers were too low to reach the level of significance.

As the list of microorganisms being associated with a variety of cancers continues to grow, we performed a literature review to summarize the epidemiological studies addressing the association between bacteria and parasites and cancers in the gastrointestinal tract (**chapter 7**). Overall, we provided an overview of the study designs and main findings of 158 studies covering 10 bacteria and three parasites. Most studies addressed the association between bacteria and colon or colorectal cancer. Many of these compared the presence or abundance of a bacterium in cancer patients *versus* healthy controls or tumor tissue *versus*

off-tumor tissue, which does not allow for assessing cause-effect relationships. A relative small portion of studies included a follow-up time of several years.

In **chapter 8,** we aimed to further unravel a causal link between *Salmonella* infection and colon cancer from a bacterial perspective. We therefore performed a 'case-control study' using 60 *Salmonella* isolates selected based on the linkage of national *Salmonella* surveillance and cancer diagnosis data in the Netherlands. Indeed, through this linkage, 30 isolates were identified that were obtained from patients with a *Salmonella* infection who developed proximal colon cancer >1 year after the infection (i.e., case isolates), and another 30 matched isolates (based on *Salmonella* serovar, gender, age and calendar year) were obtained from patients who were not diagnosed with colon cancer (i.e., control isolates). All these isolates were sent to the Dutch National Institute for Public Health and the Environment (RIVM) for typing as part of the national (human) salmonellosis surveillance. The selected 60 *Salmonella* isolates were used in several analyses. No difference in infectivity was observed between case isolates and control isolates in a cell model resembling the human gastrointestinal tract. On the other hand, we found substantial variation between isolates in terms of their capacity to infect MEFs and to induce cellular transformation, with a tendency towards higher transformation efficiency in the case isolates. Whole-genome sequencing of the isolates did not reveal biologically relevant genes or single nucleotide polymorphisms significantly associated with transformation efficiency. Assessing the capacity of the isolates to utilize a broad range of carbon, nitrogen, phosphorus and sulfur sources showed a significant positive correlation between transformation efficiency and utilization of a range of sources, mainly amino acids, peptides and phosphorus sources. Isolates with a larger metabolic flexibility in response to nutrient availability possess a biological advantage during human infection. However, the implications of these findings for *Salmonella*'s putative tumorigenic potential and which pathways/mechanisms might be involved need to be further unraveled.