Title: *In vivo* evaluation of apocynin for prevention of *Helicobacter pylori*-induced gastric carcinogenesis

Running title: Apocynin to prevent *H. pylori*-induced cancer

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ABSTRACT

Background: The emergence of antibiotic-resistant *H. pylori* strains jeopardizes the efficacy of eradication therapy and encourages the development of alternative treatment strategies. Apocynin inhibits neutrophil NADPH oxidase and could therefore decrease reactive oxygen species-mediated tissue damage in *H. pylori*-infected stomach tissue.

Materials and methods: Apocynin was tested *in vitro* for its cytotoxic and direct antibacterial effects. The therapeutic efficacy of orally administered apocynin (100 mg/kg/day via drinking water or 200 mg/kg/day via combined administration of drinking water and slow-release formulation) was assessed at 9 weeks post-infection in the Mongolian gerbil model. Bacterial burdens were quantified by viable plate count and qPCR. Histopathological evaluation of antrum and pylorus provided insight in mucosal inflammation and injury.

Results: Apocynin showed no cytotoxic or direct antibacterial effects *in vitro* or *in vivo.* Nine weeks of apocynin treatment at 200 mg/kg/day reduced active *H. pylori* gastritis, as neutrophil infiltration in the mucous neck region and pit abscess formation significantly decreased.

Conclusions: In our gerbil model, prolonged, high-dose apocynin treatment significantly improved *H. pylori* gastritis without indications of drug toxicity. Our promising results encourage further investigation of the dosage regimen and formulation and the long-term impact on neoplastic development.

Keywords: *Helicobacter pylori*, apocynin, gastric ulcer, gastric abscess, gastric cancer, treatment

INTRODUCTION

Helicobacter pylori is a Gram-negative bacterium that resides in the gastric mucosa of about half the world population (Dunn et al., 1997). Despite the acute inflammatory reaction upon colonization, the immune system cannot clear *H. pylori* infection, since it evades and modulates the primary immunological responders (Muller et al., 2011; Rubin and Trent, 2013). While neutrophils and macrophages have the capacity to phagocytize and kill most bacterial invaders, *H. pylori* deploys an arsenal of mediators, such as superoxide dismutase (SodB), catalase (KatA) and alkyl hydroperoxide reductase (AhpC), to protect itself against reactive oxygen species (ROS) and antibacterial proteins in the phagosome (5,6). In addition, *H. pylori* directs NADPH oxidase (NOX) targeting towards the cell membrane, which results in the massive release of superoxide radicals ($O_2^{\bullet -}$) in the extracellular space. As such, persistently infected stomach tissue is continuously exposed to cytotoxic bacterial virulence factors and ROS (Allen and McCaffrey, 2007). Depending on multiple bacterial, immunological and environmental factors, *H. pylori* infection leads to a variety of clinical outcomes, ranging from asymptomatic chronic gastritis over gastric ulcers to gastric carcinoma or lymphoma (Allen and McCaffrey, 2007; Hardbower et al., 2013). Since 10 to 20% of *H. pylori*-infected individuals develop significant morbidity, current treatment guidelines aim to clear the infection immediately after diagnosis using a combination of two antibiotics and a proton pump inhibitor (Malfertheiner et al., 2012). However, emerging antibiotic resistance threatens this treatment paradigm and highlights the need for alternative therapeutic options (Ayala et al., 2014).

Apocynin or acetovanillone is a non-toxic, natural organic compound that has been used extensively in Oriental medicine for a variety of conditions (Xu et al., 2014). Besides the direct inhibition of neutrophil NADPH oxidase, apocynin prevents the activation of NF-κB, which is an important mediator of gastric inflammation (Barnes and Larin, 1997; Lafeber et al., 1999). Apocynin has already been preclinically investigated to tackle inflammatory disorders, such as Parkinson's and Alzheimer's disease, and proved to inhibit airway inflammation in a clinical trial with asthma patients (Lull et al., 2011; Ghosh et al., 2012; Stefanska et al., 2012). This study assessed the anti-inflammatory activity of apocynin in the Mongolian gerbil model for *H. pylori*-induced gastric disease.

METHODS

1 Apocynin

Apocynin was purchased from Sigma-Aldrich (≥ 98%, Diegem, Belgium). To obtain slow-release (SR) floating microspheres, apocynin was added to molten stearic acid (Mosselman, Ghlin, Belgium), homogenized and cooled (Umamaheshwari et al., 2003). Thereafter, the solidified matrix was ground and the fraction of particles smaller than 300 µm was isolated. The ratio of apocynin/stearic acid in the spheres was 30%.

2 *H. pylori* culture

The highly virulent Sydney strain 1 (SS1) (kindly provided by Sara Lindén, Gothenburg University, Sweden), which is CagA- and VacA s2m2-positive, was grown for 48 hours on Tryptic Soy Agar (TSA) (Lab M Limited, Lancashire, UK) plates, supplemented with 5% sheep blood (SB) (Oxoid, Cambridge, UK). The plates were incubated at 37°C under micro-aerophilic conditions (5% O_2, 10% CO_2, 85% N_2), generated by a Whitley H35 Hypoxystation (Don Whitley, West Yorkshire, UK) and transferred to liquid Tryptic Soy Broth (TSB) (Lab M Limited, Lancashire, UK) containing 10% inactivated fetal calf serum (iFCS) (Invitrogen, Ghent, Belgium), 24 hours before inoculation of the gerbils.

3 Antibacterial effect of apocynin on *H. pylori*

The minimal inhibitory concentration (MIC) of apocynin was evaluated using agar dilution according to the Clinical Laboratory and Standards Institute (CLSI) guidelines (Standards., 2006). The test concentrations ranged from 0.5 to 256 µg/ml.

4 Cytotoxicity of apocynin on MRC-5 cells

Assays were performed in 96-well microtiter plates, each well containing 1 x 10^4 MRC-5 cells/well. After 72 hours incubation with the test compound (0.244 nM – 64 µM), cell viability was assessed fluorimetrically after addition of resazurin (Sigma-Aldrich, Diegem, Belgium) (λ_{ex} 550 nm, λ_{em} 590 nm). The results were expressed as % reduction in cell growth/viability compared to untreated control wells.

5 Animals

Female Mongolian gerbils (5 weeks old, 45-50 g; Charles River, Leiden, The Netherlands) were housed per two at 20°C and 50% humidity with a light⁄dark cycle of 12⁄12 hours and food and sterilized drinking water *ad libitum* (Carfil Quality, Turnhout, Belgium). The body weight and behavior of the animals were recorded to detect drug toxicity. The ethical committee of the University of Antwerp approved the design and procedures of the animal experiment.

6 *In vivo* experimental design

Table 1 shows the control and treatment groups to which 35 gerbils were randomly assigned. Groups 1 and 2 served as uninfected controls and were given sterilized tap water without or with apocynin, respectively (Table 1). At the start of the experiment (day 0), groups 3 through 6 were infected with 0.1 ml of TSB (Lab M Limited, Lancashire, UK) containing 1 x 10^9 CFU *H. pylori*/ml by oral gavage at two time points with an interval of one hour. This procedure was repeated after 48 hours. The gerbils were fasted from 4 hours before infection until 4 hours after gavage. The uninfected controls (1 and 2) were only given TSB.

Immediately after *H. pylori* infection, the gerbils of the treated groups (4 and 6) were exposed to apocynin dissolved in their drinking water. The total daily water intake of Mongolian gerbils was estimated at about 8 to 13% of their bodyweight (McManus, 1972). For gerbils of about

60 g, the daily dose of 100 mg apocynin/kg bodyweight could be attained by dissolving 1.538 g apocynin in 2 l sterilized and preheated (60°C) tap water (0.769 g/l). This volume was divided over 10 drinking bottles. The drinking water was refreshed 3 times per week and the weight of the bottles was monitored to estimate the amount of water consumed. Group 6 received a supplementary dose of an apocynin/stearic acid (SA) slow release (SR) formulation (100 mg apocynin/kg in 0.1% NaCMC, once daily, 5 days per week) to maximize exposure to the compound. Group 5 served as an SA gavage control and received only SA in 0.1% NaCMC, following the same schedule.

Nine weeks after infection with *H. pylori*, gerbils were killed by CO_2 overdose for aseptic removal of the stomach. The tissue was weighed, opened along the greater curvature to obtain 2 equal parts and washed with PBS. One half was divided longitudinally and subjected to (1) DNA extraction for real-time quantitative PCR (qPCR) and (2) homogenization to cultivate *H. pylori.* The other half was also split into two sections for histopathological examination.

7 DNA extraction from stomach tissue

One quarter of the stomach tissue was weighed, cut into small pieces using a scalpel blade and collected in an Eppendorf tube. DNA extraction was performed using the QIAamp DNA Mini kit (QIAgen, Venlo, The Netherlands) according to the manufacturer's instructions.

8 qPCR quantification of *H. pylori*

Sybr Green-based qPCRs for the *H. pylori* 16s rRNA gene were performed as described by Yamazaki *et al* (2005) (Yamazaki et al., 2005) in a StepOnePlus™ Real-Time PCR system (Applied Biosystems, Foster City, California, USA). Each 20 µl reaction mixture contained 10 µl of Power SYBR® Green PCR Master Mix (Applied Biosystems), 4 pmol of each primer, 5 µl of the DNA sample and PCR water (Sigma-Aldrich). A 10-fold serial dilution series of *H. pylori* DNA (11.99 – 11.99 x 10^5 fg/µl) was used to construct the standard curve for quantification of the bacterial burden. Three technical replicates were analyzed for each sample. Thermal cycling conditions involved an initial denaturation step of 95°C for 10 min and 40 cycles at 95°C for 15 sec (denaturation) and 60°C for 1 min (annealing and elongation).

9 *H. pylori* cultivation from stomach tissue

One quarter of the stomach tissue was weighed, collected in 1 ml TSB and homogenized (TissueRuptor®, QIAgen). The homogenate was immediately placed in micro-aerophilic conditions (5% O_2, 10% CO_2, 85% N_2) in a Whitley H35 Hypoxystation (Don Whitley, West Yorkshire, UK) and serially diluted for viable plate counting (VPC). Dilutions were spread onto TSA plates, supplemented with 5% sheep blood (Oxoid, Cambridge, UK), vancomycin (10 µg/ml), trimethoprim (5 µg/ml), amphotericin B (5 µg/ml) and cefsulodin (10 µg/ml) (all purchased from Sigma-Aldrich) and incubated at 37°C under micro-aerophilic conditions for 5 to 10 days.

10 Histopathological evaluation

Tissue sections were stretched on Sylgard® 184 (Sigma-Aldrich) in a petri dish and fixed for 1 hour in 4% paraformaldehyde (PAF, Sigma-Aldrich) in PBS at room temperature. The tissue was rinsed 3 times for 10 min with PBS and stored in PBS + 0.01% NaN_3 at 4°C. After fixation, the tissue was processed in a Citadel 2000 (Thermo Fisher Scientific, Aalst, Belgium) and embedded in paraffin. Tissue sections of 5 µm were cut and stained with H&E. Subsequently, an experienced pathologist scored the sections blindly for three different parameters (Table 2).

RESULTS

1 Apocynin does not inhibit *in vitro* growth of *H. pylori*

After 5 days of *in vitro* culture, all *H. pylori* colonies showed the same size and morphology on the agar plates containing different concentrations of apocynin. Since apocynin had no inhibitory effect on the growth of *H. pylori*, no MIC value could be determined (MIC > 256 µg/ml).

2 Apocynin does not affect cell viability up to 64 µM

Fluorimetric assessment of MRC-5 cells after resazurin staining indicated that the cell viability and growth of apocynin-treated cells were comparable to that of the untreated controls, confirming the non-cytotoxic nature of the compound (IC_{50} > 64 µM).

3 Apocynin treatment does not affect *H. pylori* burden in the gerbil stomach

The load of *H. pylori* per gram stomach tissue was determined by qPCR, detecting all *H. pylori* DNA in the stomach, and VPC, quantifying all viable *H. pylori*. The uninfected control groups (1 and 2, data not shown) had no *H. pylori* in their stomach, while all infected groups (3 to 6) showed similar bacterial burdens for both qPCR and VPC (Kruskal-Wallis, n=4 or 6, p>0.05) (Figure 1).

4 Exposure to apocynin in DW failed to influence *H. pylori*-induced inflammation

H&E-stained tissue sections were histologically assessed to identify the gastric region on the slide. For each animal, slides containing antrum and pylorus were blindly scored (Table 2). The stomach tissue of the animals in groups 1 or 2 showed normal histology without signs of inflammation. Immunohistochemical staining using an *H. pylori*-specific antibody confirmed the presence of the bacterium in all infected gerbils, whereas no *H. pylori* bacteria were seen in the uninfected groups.

Figure 2 shows that exposure of infected gerbils to DW with a concentration of 0.769 g/l apocynin did not influence the inflammatory progression of *H. pylori* infection. Compared to the infected control group (3), neutrophil influx or abscess formation in the antrum and

pylorus region remained unaltered upon apocynin exposure (group 4, Chi-square, n=6, p>0.05). In addition, the same levels of lymphocytic infiltration were observed (Chi-square, n=6, p>0.05).

5 Treatment with SR apocynin significantly decreased pit abscess formation

Figure 3 shows that treatment with SR apocynin ameliorated *H. pylori*-induced gastritis, as less pit abscess formation was observed in the treated group (6) compared to the SA gavage controls (group 5, Chi-square, n=4 and 7, p=0.044). The influx of lymphocytes was comparable in both groups (Chi-square, n=4 and 7, p>0.05).

DISCUSSION

This study evaluated the effect of apocynin on *H. pylori*-induced inflammation. In the immune response to *H. pylori* colonization of the human stomach, neutrophils provide the first line of defense (Guang et al., 2012). The production of ROS, proteolytic enzymes and antimicrobial peptides form the basis of the neutrophil's protective capacity, but these molecules do not selectively destruct invading microbes (Handa et al., 2010). The combination of neutrophil ROS and bacterial virulence factors causes collateral damage to the gastric epithelium (Cadamuro et al., 2014). Since apocynin directly inhibits $O_2^{\bullet-}$-producing NADPH oxidase of neutrophils and macrophages, this natural compound could prevent cytotoxic ROS formation and subsequent gastric tissue injury (Stefanska and Pawliczak, 2008).

Successful *in vivo* studies using anti-inflammatory molecules, such as capsaicin and irsogladine, to tackle *H. pylori*-induced disease encourage the concept of non-antimicrobial therapeutic approaches, i.e. the modulation of the pathogenesis without bacterial eradication (Akagi et al., 2013; Mozsik, 2014). The promising reports of apocynin treatment of several inflammatory disorders triggered us to explore its effect on gastric injury due to *H. pylori* infection. In a mouse model of zymosan-induced acute arthritis, oral administration of apocynin significantly improved proteoglycan synthesis in the knee-joint (Hougee et al., 2006). Apocynin treatment in a marmoset model of Parkinson's disease mitigated the disease progression, limiting the parkinsonian signs and motor-function deterioration (Philippens et al., 2013). Moreover, a clinical trial using nebulized apocynin demonstrated a reduction of ROS concentrations in the exhaled breath condensate of asthma patients (Stefanska *et al.*, 2012).

The existing data indicate a clear anti-inflammatory effect *in vivo*, which may be related to the direct inhibitory effects on the neutrophil's respiratory burst or the prevention of NF-κB activation. In a previous study, our research group (Horemans *et al,* 2014 – in preparation) used electron paramagnetic resonance measurements to show that apocynin significantly inhibits the respiratory burst of neutrophils stimulated with opsonized zymosan particles (OPZ). Since OPZ stimulates neutrophils through cell membrane receptor binding like microorganisms do, we considered OPZ as a proxy for bacterial activation of neutrophils. As such, our study suggests a therapeutic role for apocynin in infectious diseases in which

neutrophils contribute to the pathogenesis rather than providing protection to the host. This is the case for *H. pylori*-induced gastric inflammation, but also for disease caused by *Mycobacterium tuberculosis* (Eruslanov et al., 2005). Localized treatment with apocynin could prevent the generation of extracellular release of ROS and an important part of the associated tissue damage (Kennett et al., 2011).

This study evaluated the therapeutic efficacy of apocynin in the gerbil model for *H. pylori* infection, because its inflammatory course is comparable to that in humans. Since apocynin is expected to influence neutrophil activity, mouse models, which are characterized by mononuclear cell infiltration instead of neutrophil influx, were considered less suitable (Zhang and Moss, 2012). Infection of the gerbil stomach with *H. pylori* SS1 leads to the recruitment of inflammatory cells from 3 weeks post-infection and results in histopathologically well-described gastric disease over a period from 4 to 64 weeks post-infection (Wiedemann et al., 2009) (Elfvin et al., 2005; Chandan et al., 2013). In the antrum, severe active gastritis can be observed at 8 weeks post-infection, whereas the corporal inflammation gradually increases over time. Early precancerous conditions, such as atrophy, metaplasia and dysplasia, start to develop from 8 weeks post-infection (Wiedemann *et al.*, 2009). *H. pylori* SS1 is an enthusiastic colonizer of the gerbil stomach, which is reflected by our 100% infection rate and assures the establishment of a reproducible animal model.

Since this study was the first to explore the effect of apocynin on the clinical outcome of *H. pylori*-infection, the dosing regimen was designed to obtain the highest possible concentration and exposure time in the stomach. The absence of cytotoxicity in earlier studies and in our own *in vitro* assessment encouraged us to use high doses of approximately 100 mg/kg apocynin via the drinking water and 200 mg/kg when the drinking water was supplemented with the daily administration of the slow-release formulation of apocynin (de Almeida et al., 2011; Pedroso et al., 2013). In addition, apocynin administration was started at the time of infection and continued until 9 weeks post-infection, when histopathological signs of active gastritis become apparent in untreated animals. As a water-soluble compound, apocynin allowed oral administration via drinking water. This mode of administration offers a comfortable way of treating animals for a longer period of time and directs the compound immediately to the desired site of action. To further maximize exposure and to compensate for the limited uptake during the night due to the circadian drinking pattern, one treatment group (6) received an SR formulation of apocynin and SA (Rodriguez et al., 1999).

Nine weeks after infection and initiation of treatment, the stomachs were excised for in-depth morphological and microbiological evaluation. The qPCR and VPC data on bacterial burden were in line with our *in vitro* finding that apocynin treatment has no effect on *H. pylori* viability, growth or attachment (Figure 1).

Histopathological analysis was based on tissue sections containing the gastric regions antrum and pylorus, because they consistently show a high infectious load and severe gastritis at 9 weeks post-infection in control animals. Immunohistochemistry confirmed the presence of *H.*

pylori in all infected gerbil stomachs (Figure 2). Untreated (Group 1) and treated (Group 2) uninfected animals showed no *H. pylori* and normal gastric histology, indicating that apocynin has no direct effect on the stomach tissue. For the infected animals, the administration of apocynin via DW (Group 4) did not affect neutrophil influx and abscess formation. However, the combined administration of apocynin in DW and SR formulation (Group 6) significantly decreased pit abscess formation in the mucous neck region compared to the SA control group (Group 5). This interesting finding suggests that apocynin has the capacity to positively modulate the inflammatory process and may thus *H.pylori*-induced tissue damage.

Given the small study size and the importance of the dosage strategy, additional experiments in the gerbil model are required to gain full insight in the therapeutic abilities of apocynin. First, assessment of its effect on neoplastic development at 32 to 64 weeks post-infection could convincingly demonstrate its applicability in gastric disease. Equally important is the careful optimization of the daily dosage and formulation approach. Since we hypothesized that apocynin should influence the activity of neutrophils that assemble in the stomach upon *H. pylori* colonization, we aimed to maximize the exposure of these immune cells. In this exploratory study, apocynin in DW and as SR formulation were combined to compensate for (1) the dilution effect of the stomach fluid, (2) the limited retention time due to gastric emptying and (3) the challenging diffusion towards neutrophils that are hidden in the stomach mucosa (Harsha, 2012). Possibly, higher dosages are even more effective to inhibit the ROS production, but caution is warranted as Tang *et al* highlighted the delicate balance between the pro- and anti-inflammatory effects of apocynin treatment (Tang et al., 2008). The narrow therapeutic window that was reported for the treatment of experimental stroke is supported by pro-oxidant activity of high doses *in vitro* (Castor et al., 2010; Connell and Saleh, 2012).

The optimal dosage regimen for efficacy in the stomach should be based on apocynin concentrations in the gastric mucosa (Gustavson et al., 1995). A recent pharmacokinetic study on plasma levels demonstrated a short half-life ($t_{1/2}$= 6 min) and limited bioavailability for oral administration of apocynin to rats (Wang et al., 2013). While these compound characteristics complicate systemic treatment with apocynin, the short half-live supports our localized and continuous treatment strategy. The SR formulation allowed prolonged exposure of the mucosal immune cells without accumulation of the compound in the circulation. Modification of the drug delivery mode, e.g. using mucoadhesive or mucopenetrating systems, could further enhance the gastric retention time (Conway, 2005; Arora et al., 2011; Zhao et al., 2014).

In conclusion, this is the first study to explore the therapeutic effect of apocynin on *H. pylori*-induced inflammation. In our gerbil model, prolonged, high-dose apocynin treatment significantly reduced pit abscess formation without indications of drug toxicity. Our promising results provide incentive to further investigate the dosage regimen and formulation and study the long-term impact on neoplastic development.

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TABLES

Table 1. Overview of the different test groups for the *in vivo* evaluation of apocynin[1]

Group	Abbr.	#	*Hp*	Treatment	Purpose
1	-C	6	No	None	Negative control
2	$-C_{APO}$	6	No	Apocynin DW	Apocynin control
3	C_{DW}	6	Yes	None	Infected control
4	$T_{APO\ DW}$	6	Yes	Apocynin DW	Apocynin DW treatment
5	C_{SA}	4	Yes	SA gavage daily	SA gavage control
6	$T_{APO\ SR}$	7	Yes	Apocynin DW + gavage SR daily	Apocynin SR treatment

Table 2. Histopathological scoring system for the evaluation of H&E-stained tissue sections of the gerbil stomach

Parameter	Score	Description
Infection	0	No *H. pylori*
	1	*H. pylori*
Lymphocytes	0	No or little lymphocytes present
	1	Infiltration of lymphocytes at mucosal base
	2	Lymphoid follicles (follicular gastritis)
Neutrophils	0	No or little neutrophils present
	1	Neutrophil infiltrate in mucous neck region
	2	Pit abscess formation

FIGURES

Figure 1. *H. pylori* bacteria per gram stomach tissue, determined by qPCR (left) and viable plate count (VPC) (right). No statistically significant differences were found between the untreated and treated groups (Kruskal-Wallis, n=4-6, $p>0.05$). C: Control, T: Treatment, DW: Drinking Water, APO: Apocynin, SA: Stearic Acid, SR: Slow Release.

Figure 2. Histopathological scoring of neutrophil influx in stomach tissue of gerbils treated with apocynin supplemented drinking water versus vehicle controls (Chi-square, n=6, $p>0.05$). C: Control, T: Treatment, DW: Drinking Water, APO: Apocynin.

Figure 3. Histopathological scoring of neutrophil influx in stomach tissue of gerbils treated daily with a slow-release formulation of apocynin and apocynin supplemented drinking water versus vehicle controls (Chi-square, n=4-7, $p=0.044$, indicated by *). C: Control, SA: Stearic Acid, T: Treatment, APO: Apocynin, SR: Slow Release.

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Figure 1

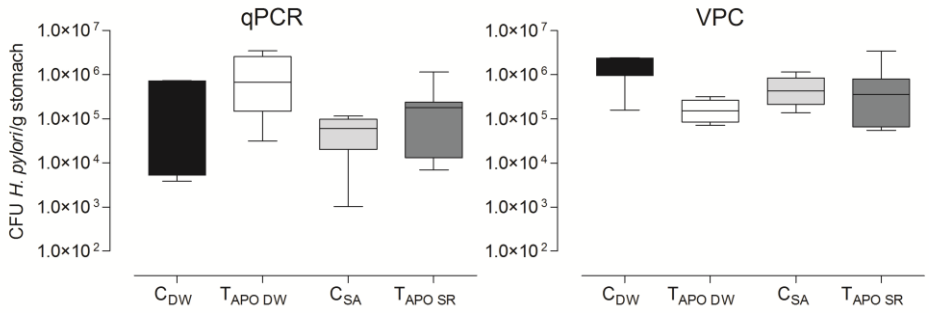

Figure 2

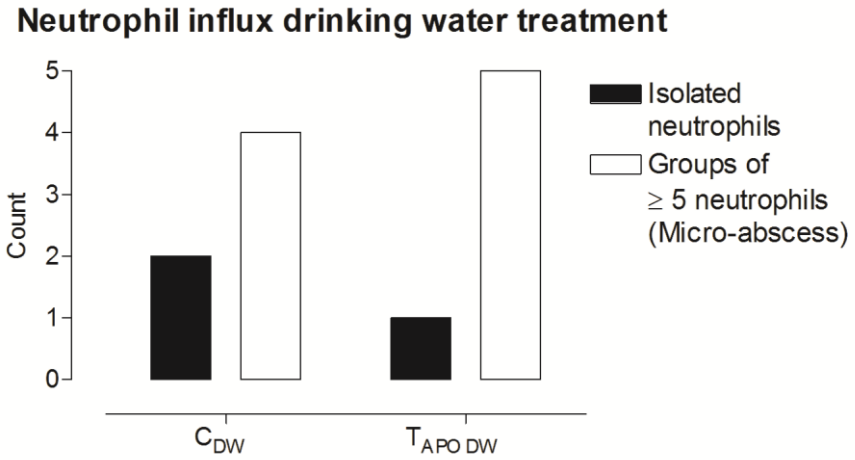

Figure 3