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The jejunum is the main site of absorption for anthocyanins in mice

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Abstract

Intestinal absorption of anthocyanins (ACNs) was studied in vitro by comparing ACN disappearance from the mucosal solution of Ussing chambers not containing any tissue (controls) and that of Ussing chambers containing segments of mouse duodenum, jejunum, ileum or colon. The tissues were mounted in the chambers and bathed with Ringer's solution (RS) adjusted to a pH representative of the respective segments in vivo. The chambers were kept at 37°C and RS was perfused continuously with carbogen (95% O_2/5% CO_2). After the addition of an ACN extract to the mucosal solution, samples from both the mucosal side and the serosal side were withdrawn at 10, 40, 80 and 120 min and analyzed for ACN concentration using reversed-phase HPLC with photodiode array detection. The highest absorption of ACNs occurred in chambers mounted with jejunal tissue (max absorption rate, 55.3±7.6%). Minor absorption occurred with duodenal tissue (10.4±7.6%), with no absorption recorded when tissues from the ileum or colon were used. This study demonstrates for the first time that ACN absorption in mice occurs predominantly in the jejunum.

Keywords: Anthocyanins; Ussing chamber; Absorption; Intestinal segments

1. Introduction

Anthocyanins (ACNs) are responsible for the red, purple and blue colors displayed by many vegetables and fruits (particularly berries) and are part of a large and widespread group of water-soluble plant constituents known collectively as flavonoids [1]. Their intake in humans has been estimated to be as much as 180–215 mg/day in the United States [2] due to their widespread distribution and occurrence in fruits and vegetables. Berry fruits in particular are rich dietary sources, and some can contribute 100–300 mg of ACNs in a single serving [3]. As a potential major component of our daily diet [2], increasing attention has focused on the biologic activities and possible health benefits of ACNs. In particular, the antioxidant properties of flavonoids [4] are thought to protect cells against oxidative damage that contributes to various pathologies, including diabetes [5], arteriosclerosis [6], neurodegeneration [7] and some cancers [8–10]. However, to evaluate the health effects of ACNs in detail, their bioavailability, including absorption, metabolism and excretion, must be known [11]. At present, these aspects of ACNs are still not fully understood.

Most studies have found that the glycosidic forms of ACNs are absorbed intact and appear in plasma and urine samples [3,12–17], whereas other studies have found metabolites of ACNs in urine as well as in tissues such as the liver and kidneys [11,12,17,18]. Nevertheless, the fate of ACNs within the gastrointestinal tract (GIT) is largely unknown.

pH has a marked influence on the color and stability of ACNs in aqueous media [19,20] where ACNs exist as a mixture of at least four molecular species: the colored basic flavylium cation and three secondary structures, the quinoidal base, the carbinol pseudobase and the chalcone pseudobase [21]. The concentration with which each compound is present in a solution is pH dependent. During the passage of ACNs through the GIT, they are exposed to different pH environments and therefore might exist as different forms. The ACN forms present in the different regions and tissues of the GIT and eventually during absorption are not known with certainty. It is likely that

Abbreviations: ACNs, anthocyanins; C3G, cyanidin-3-glucoside; CY, cyanidin; GIT, gastrointestinal tract; PDA, photodiode array; RS, Ringer's solution.

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the flavylium cation will exist only in the lumen of the stomach due to low pH and that the other forms will predominate lower down the GIT and in the epithelium if absorbed. This assumption was recently supported by the study of McGhie et al. [3]. Sixty minutes after dosing rats with a boysenberry extract, an intense red color in the stomach indicated the presence of the red flavylium cation, whereas no ACNs as the cation were observed in the small intestine. However, after acidifying the intestinal tissue, a red color appeared, indicating the conversion of colorless forms of ACNs into the red cation. It is possible that ACNs are converted to the carbinol pseudobase, quinoidal base or chalcone pseudobase during passage through the small intestine and that these compounds are the potential forms that are absorbed from the gut into the blood system [22]. Whether these forms are the ones responsible for the observed biologic activity is not known yet.

The aim of the present study was to evaluate ACN absorption at different locations in the intestine and, in particular, to identify the main absorption site within the GIT. For this purpose, Ussing chambers were used to compare the absorption (as disappearance of ACNs from the mucosal solution) of a boysenberry ACN in four intestinal segments of mice — the duodenum, jejunum, ileum and colon.

2. Methods and materials

2.1. Animals

The study was approved and followed the procedures set by the Animal Ethics Committee of the Massey University (Palmerston North, New Zealand) [23].

A total of 10 male Swiss mice (age, 6–8 weeks) were used for the experiments. The mice were obtained from the Small Animal Production Unit at Massey University. They were housed in rodent cages and kept in a room with controlled temperature (21±1°C), humidity (55±5%) and lighting (12-h light/dark cycles with dawn-and-dusk transitional periods). The mice were fed a balanced standard rodent diet (Table 1) prepared at the Food Processing Unit, Massey University and were given access to both food and demineralized water ad libitum until the day of the experiment.

2.2. Experimental setup

On the day of the experiments, mice were deprived of food for 2–3 h, anesthetized with 6% halothane and euthanized by cervical dislocation. Within 1 min of euthanasia, each mouse's abdomen was opened by a midline incision; the intestine was dissected out and immersed in Ringer's solution (RS; see below) at room temperature. Two-centimeter-long pieces of intestine from each region (duodenum, jejunum, ileum and colon) were opened longitudinally along the mesenteric border and mounted in individual Ussing chambers. The exposed surface of the tissue was 0.67 cm^2. The composition of the RS was as follows (in mmol/L): 120 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.8 NaH_2PO_4, 0.2 Na_2HPO_4, 25 $NaHCO_3$, 2 glutamine and 2 sodium pyruvate. The pH was adjusted with 1 M HCl to between 4.5 and 7.0, depending on the region from which the intestinal tissue was taken [24–28]. The tissues were kept at 37.5°C and the RS was aerated with carbogen (95% O_2/5% CO_2). All tissues were voltage clamped throughout the experiments at 0 mV [29] using automatic clamp units (Campus Electronics and Mechanical, University of Otago, Dunedin, New Zealand). The RS was replaced at least three times at the start of each experiment to wash the tissues and remove any luminal content. The volume of the RS on each side of the chambers was 8 ml. The tissues were left to equilibrate after washing for at least 30 min before the ACN extract was added to the mucosal RS.

Table 1
Ingredient composition of the diet

Ingredient	Amount (g/kg diet)
Wheat	403.5
Barley	300
Broll	50
Lucerne	50
Meat meal	60
Fish meal	70
Skim milk powder	50
Soybean oil	10
Premix[a]	5
Methionine	1
NaCl	0.5

[a] Contained (g/kg mix): Vitamin A (1.4 MIU); Vitamin D_3 (0.20); Vitamin E (10.0); Vitamin B_1 (0.80); Vitamin B_2 (1.0); Vitamin B_6 (1.2); Vitamin B_{12} (0.004); Vitamin B_5 (5.0); biotin (0.02); Vitamin B_3 (4.0); folic acid (0.20); cholin (50.0); Fe (16.0); Zn (10.0); Mn (10.0); Cu (1.0); I (0.10); Co (0.14); Sn (0.04); Mg (100.0); Ca (147.8); antioxidant (0.026); K (100.1); S (10.3); and Cl (9.1).

Chambers without tissue were used as controls to measure ACN degradation due to experimental conditions during the 120 min of the experiment. Therefore, for each intestinal region, control chambers were set up and bathed with an RS of the same pH value as the respective intestinal segment.

2.3. ACNs

Frozen boysenberries (*Rubus loganobaccus* × *baileyanus* Britt, 600g) were extracted twice with 1.5 L of acetone/water/acetic acid (70:29.5:0.5). Extracts were evaporated to a 500-ml total volume at 40°C and lipids were removed by extraction (three times) with 500 ml of hexane. The aqueous layer was evaporated and then diluted to 20% methanol/water using methanol. Subsequently, the aqueous fraction was applied onto a large LH-20 Sephadex column (50-mm ∅×370-mm height; 450 g LH-20) and washed with 20% methanol/water. Boysenberry ACNs were eluted with 60% methanol/water, evaporated to dryness and stored in the dark at 4°C until use.

ACNs of boysenberries have previously been characterized as cyanidin-3-sophoroside, cyanidin-3-glucoside (C3G), cyanidin-3-glycosylrutinoside and cyanidin-3-rutinoside [17,30]. The extract used in the present study contained predominantly C3G (Fig. 1), the major monosaccharide ACN of boysenberries [17]. For each experiment, a stock solution containing 5 mg of boysenberry extract per 4 ml of RS at the appropriate pH value was prepared. This stock solution was used to prepare a standard for each pH value as well as application of the extract to the mucosal solution (8 ml) with an average C3G concentration of 2.67±0.15 μmol/L.

2.4. Sample preparation

Samples (400 μl) from the mucosal and serosal compartments of the Ussing chambers were taken at 10, 40, 80 and 120 min. Mucosal samples were immediately acidified (1:1) with 5% formic acid/H_2O and stored at 4°C until analysis. Serosal samples were stored at 4°C until solid-phase extraction was performed, usually not later than 24 h. IST Isolute C18 (EC) cartridges were conditioned with 5% formic acid/methanol (5 ml) and 5% formic acid/H_2O (10 ml) before the serosal samples (400 μl) were loaded onto the columns. Subsequently, the cartridges were washed with 5% formic acid/H_2O (10 ml) and ethyl acetate (5 ml) before the columns were dried by drawing air for approximately 15 s to remove ethyl acetate. ACNs were eluted into glass tubes with 5% formic acid/methanol (2 ml). The extracts were evaporated close to dryness under a stream of N_2 at ≤35°C and the residue was redissolved in 5% formic acid/H_2O (200 μl). Samples were transferred into HPLC vials and stored at 4°C until analysis.

2.5. HPLC analysis

C3G concentrations in mucosal and serosal samples were determined by reversed-phase HPLC with photodiode array detection (PDA). The column used was a LiChrosphere 100 RP-18 end-capped 5-μm column (250×4 mm). Solvents A (1.5% *o*-phosphoric acid) and B (acetic acid/acetonitrile/phosphoric acid/H_2O, 20:24:1.5:54.5) were run at a flow rate of 1 ml/min. The solvent gradient started with a composition at 80% A–20% B and changed to reach 30% A–70% B at 25 min. The composition then changed to reach 10% A–90% B by 30 min and held this composition until 35 min before returning to the starting conditions at 40 min for 5 min, with a total run time of 45 min. The sample injection volume was 10 μl for the mucosal samples and 50 μl for the serosal samples. The PDA was used to collect spectral data (250–600 nm) and chromatograms were extracted at 520 nm. Chromatography data were collected and processed using a Water Millennium Chromatography Manager (Version 4.0). C3G concentrations were calculated using an authentic standard of cyanidin-3-*O*-galactoside with known concentration.

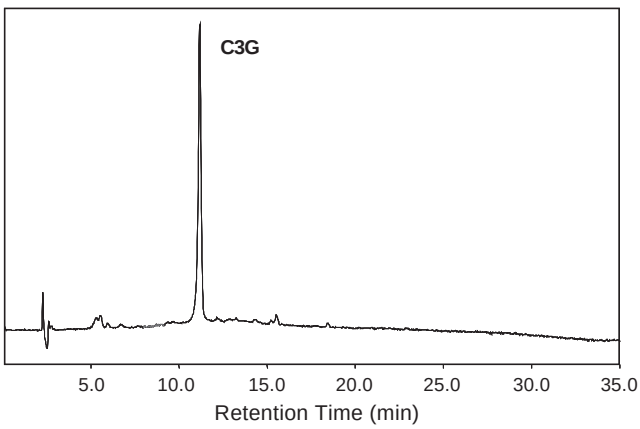

Fig. 1. Representative HPLC chromatogram of purified boysenberry C3G.

2.6. Statistical analysis

Data are presented as means±S.E.M. Statistical analysis was carried out using SAS System for Windows (Version 8). The residues of each analysis were tested for normality. The significance of differences was assessed by one-way ANOVA for comparison of individual means. Differences with $P \leq .05$ were considered significant.

3. Results

After adding C3G to the RS of the mucosal compartment of the Ussing chambers, C3G disappearance from the mucosal solution and C3G appearance in the serosal solution were measured by HPLC. Fig. 2A–D shows C3G concentrations in the mucosal solution of tissue-mounted chambers together with each respective control during the 120 min of the experiment.

With the duodenal segment, a significant decrease of C3G over time could be seen with the intestinal tissue (27.6%), whereas there was no significant effect over time with the control throughout the experiment (Fig. 2A). Significant differences between the control and the intestinal tissue, indicating absorption, were observed at 80 and 120 min.

For the jejunal tissue, there was a significant decrease in C3G concentration in the mucosal solution over time (initial concentration, 1.26 μg/ml; concentration after 2 h, 0.57±0.03 μg/ml=54.8%) (Fig. 2B). This decrease was not due to degradation as the control showed that C3G was stable in RS throughout the experiment (concentration 2 h after mucosal addition, 1.23±0.03 μg/ml=98.6%). A significant difference ($P<.001$) between the control and the jejunal tissue, indicating absorption, appeared already after 10 min.

With the ileal and the colonic segments, both the controls and the respective intestinal tissues showed a significant C3G decrease over time (ileum–control, 31.8%; ileum–tissue, 34.8%; colon–control, 16.5%; colon–tissue, 18.2%)

(Fig. 2C and D). However, no significant difference between the controls and the intestinal tissues, representing absorption, was observed at any time point.

C3G was not detected in any serosal solution (data not shown), which implies that the adjacent tissue layers that were still present in preparations may have hindered diffusion across the tissue.

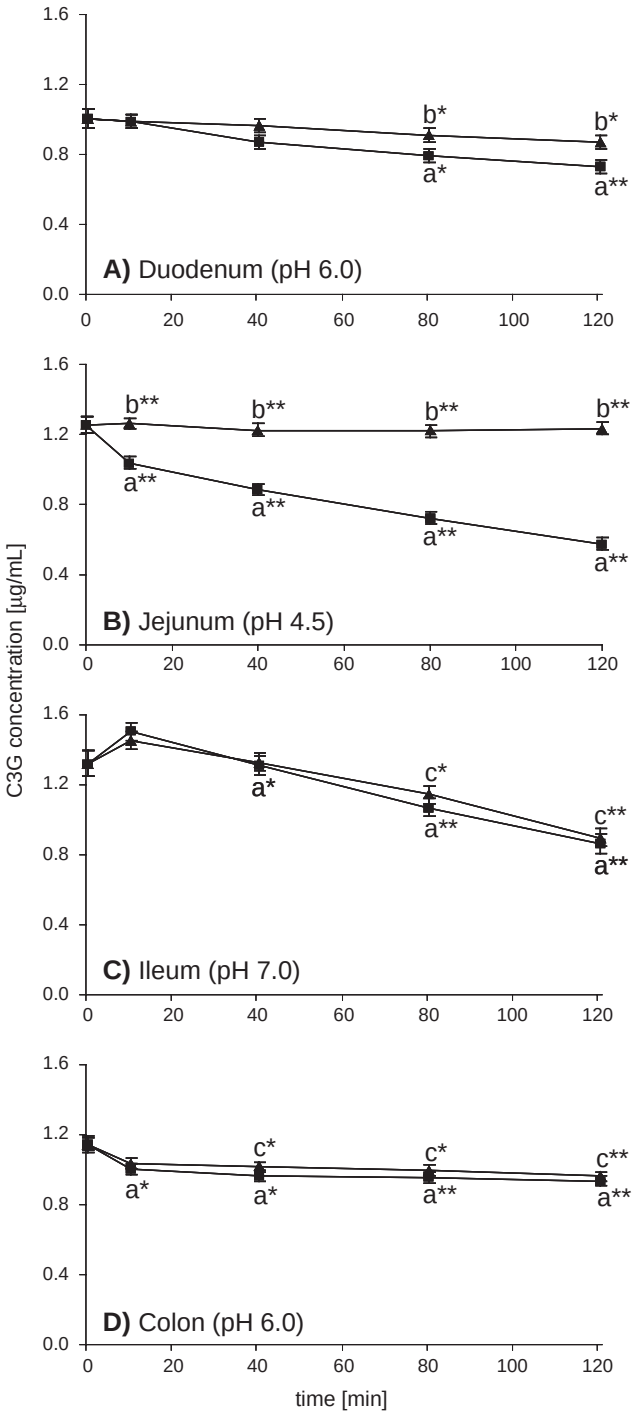

Fig. 2. C3G concentration over time in mucosal solution of Ussing chambers mounted with duodenal (A), jejunal (B), ileal (C), and colonic (D) tissue. (▲) Control; (■) intestinal tissue. Values are the means±S.E.M. ($n=6–8$). The letter *a* indicates significant difference over time (intestinal tissue); *b*, significant difference between intestinal tissue and control at the respective time point; *c*, significant difference over time (control). *$P<.05$; **$P<.001$.

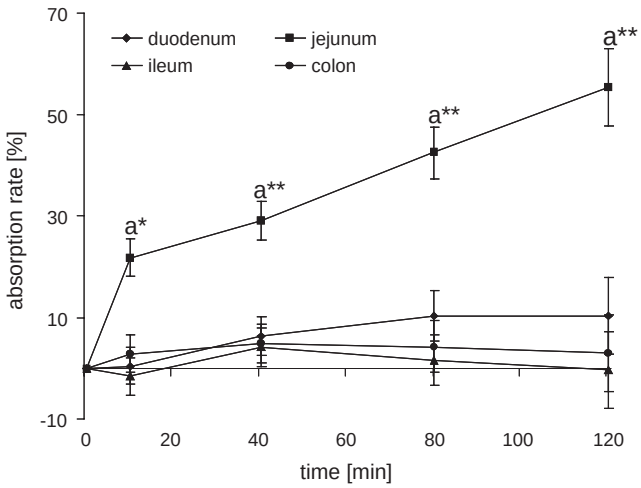

Fig. 3. C3G absorption rate over time. Values are the means±S.E.M. ($n=8$). The letter *a* indicates the significant difference between the jejunum and the other intestinal segments at the respective time point. *$P<.01$; **$P<.001$.

The differences between C3G degradation with each control and C3G decrease with the respective intestinal tissue (as seen in Fig. 2A–D) were calculated and labeled as ACN absorption. The C3G absorption rate over time is shown in Fig. 3. The jejunal segment showed a linearly increasing C3G absorption over time, with a maximum absorption rate of 55.3±7.6% at 2 h. Its absorption rate was significantly higher at each time point compared with the duodenal, ileal and colonic segments. A slight absorption rate of 10.4±7.6% could be seen for the duodenal segment after 80 min.

The amount of C3G that was absorbed during the 2 h of the experiment was 5.37±0.78 µg C3G/cm^2/2 h for the mouse jejunal tissue, significantly higher than that for the other intestinal segments (≤1.10 µg C3G) (Fig. 4).

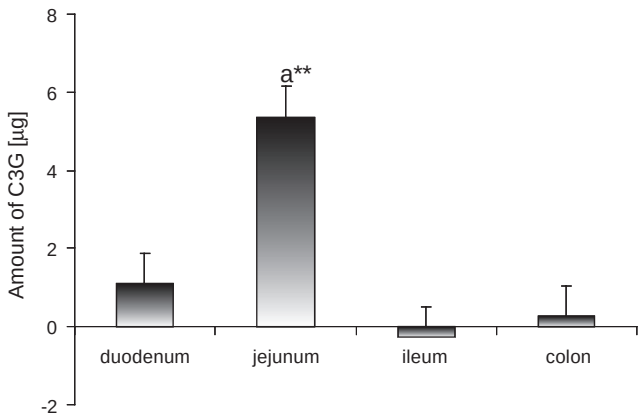

Fig. 4. Total amount C3G absorbed per cm^2 intestinal tissue (µg/cm^2/2 h). Values are the means±S.E.M. ($n=8$). The letter *a* indicates significant difference between the jejunum and the other intestinal segments. **$P<.001$.

4. Discussion

ACNs are polyphenolic compounds that belong to secondary plant metabolites and are a component of our daily diet. There has been increasing interest on their biologic activities as they are claimed to enhance health by protecting against some chronic diseases [31,32]. However, to investigate and maximize beneficial effects in humans, their bioavailability, including absorption, metabolism and excretion, needs to be understood. Although there are many studies investigating the occurrence of ACNs and their metabolites in body fluids such as blood plasma or urine, there is a lack of studies investigating the fate of ACNs within the GIT. The purpose of the present study was to investigate the absorption of ACNs from a berry fruit extract within the GIT and, in particular, to evaluate the main absorption site of ACNs using the Ussing chamber as an in vitro system and mouse intestine as the model tissue. The berry fruit extract mainly contained the ACN C3G.

We have demonstrated that C3G is absorbed from the intestine in mice at different rates depending on location within the GIT. The main absorption site for C3G was shown to be the jejunum. C3G removal from the mucosal compartment of the Ussing chamber was not due to degradation influenced by experimental conditions as the control showed that C3G was stabile throughout the experiment (Fig. 2B). The decrease in the concentration of C3G was rather due to the physiological action of the tissue and most likely indicates C3G absorption or metabolism within the respective intestinal segment. The fact that C3G absorption was found to predominantly take place in one intestinal region (i.e., the jejunum) suggests the involvement of an active transport mechanism. The hypothesis that an active transport mechanism is involved in ACN absorption is also supported by other studies. Mulleder et al. [33] found reduced excretion of ACNs in urine after simultaneous ingestion with sucrose, implying that ACN absorption is blocked by sucrose and therefore associated with intestinal sugar transport systems. Other studies have shown that monoglucosides of quercetin, a flavonoid, interact with an active transport mechanism, namely the intestinal sodium-dependent glucose transporter (SGLT1) [34,35], and are taken up into the epithelium, where they are rapidly deglycosylated and then glucuronidated [34]. Tsuda et al. [12] investigated C3G and its metabolites in jejunal tissue of rats after direct stomach intubation. C3G, the aglycone cyanidin (CY) and protocatechuic (PC) acid (oxidation product of C3G) were detected in the jejunal tissue. Tsuda et al. [12] showed that C3G is rapidly detected in plasma and that PC can be detected at concentrations eight times higher compared with C3G while CY is not present in plasma. The authors hypothesized that C3G is partly hydrolyzed by β-glucosidase to CY in the intestine. CY is unstable at physiological conditions and rapidly degraded to PC, which accumulates at high concentrations in plasma [12]. Further investigations are needed to establish if the C3G disappearance in the present study is due to metabolism along the mucus layer or to accumulation or metabolism within the epithelial tissue. Besides the jejunum, some disappearance of C3G was observed with the duodenal tissue (mean absorption rate±S.E.M., 10.4±7.6%), indicating that the duodenum also contributes to absorption or metabolism of C3G in mice. Disappearance of C3G from the Ussing chambers mounted with ileal and colonic tissues was not significantly different from their respective controls (Fig. 2C and D), indicating that no C3G absorption or metabolism occurs in the lower small and large intestines of mice.

In the present study, the acidity of the RS was adjusted to levels normally found in the intestine. In humans, the pH values in the duodenum, jejunum, ileum and colon range from 6.1 to 6.7, 4.4 to 6.6, 6.8 to 8.0 and 6.0 to 7.2, respectively [25–28]. A similar value (6.47) was found by De Lisle et al. [24] for the duodenum in mice. The acidity within the intestinal tract can vary between the fasted and fed states [27] and is most likely influenced by diet. As ACNs are pH sensitive, it was the purpose of the present study to simulate the different pH values of the different intestinal regions to investigate ACN absorption. The results show that C3G is more stable at a lower pH (Fig. 2). It is uncertain if the absorption rate in the duodenum could be increased or if any absorption could be seen in the ileum or colon if the pH of the RS is lowered to 4.5. It seems unlikely, based on the results of the present study, that lowering the pH of the RS in Ussing chambers mounted with colonic tissues would result in absorption as some absorption was observed with the duodenal tissue at the same pH (Fig. 2A and D). Nevertheless, pH values as low as 4.5 are not normally [25–28] to be expected in the ileum and colon as there is a pH gradient, with pH becoming less acidic at more distal locations [27].

Recent studies have demonstrated the possibility of ACN absorption from the stomach [36–38]. It has been suggested that an organic anion membrane carrier, bilitranslocase, expressed in epithelial cells of the gastric mucosa, could be involved in the absorption of ACNs [36]. As several studies have demonstrated that ACNs are rapidly absorbed following oral administration [12–14,39], absorption of ACNs through the gastric wall [38] may provide an explanation. pH values for the stomach have been reported to be between 1.4 and 2.1 [40]. As ACNs are more stable at a lower pH, this could play a role in ACN absorption from the stomach. Whether ACN absorption throughout the GIT is pH dependent remains to be determined.

We have demonstrated for the first time differences in C3G absorption by different intestinal segments from mice, with the highest absorption occurring in the jejunum, minor absorption occurring in the duodenum and practically no absorption occurring in the ileum or colon. The identification of the main absorption site for ACNs is a step for further investigations regarding bioavailability. Future research should focus on a possible way to maximize

absorption of ACNs and consequently to increase their beneficial health effects with a normal human diet.

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