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ORIGINAL ARTICLE

Study of the urinary and faecal excretion of N^{ε}-carboxymethyllysine in young human volunteers

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Abstract The dietary habits of the adolescent population with a high intake of snack and fast foods mean that they consume a high rate of which in turn leads to the development of different degenerative disorders. There are few studies available on MRP absorption and metabolism. We investigated the effects of a MRP-high and a MRP-low diet on carboxymethyllysine (CML) intake and excretion in 11–14 years adolescent males. In a 2-period crossover trial, 20 healthy subjects were randomly assigned to two groups. The first group consumed the MRP-low diet for 2 weeks, observed a 40-day washout period, and then consumed the MRP-high diet for 2 weeks. The second group received the diets in the reverse order. Subjects collected urine and faeces on the last 3 days of each dietary period. The consumption of the MRP-high diet led to a higher CML input ($P < 0.05$) (11.28 vs. 5.36 mg/day CML for MRP-high and -low diet, respectively). In parallel, the faecal excretion was also greater ($P < 0.05$) (3.52 vs. 1.23 mg/day CML, respectively) and proportional to the dietary intake. The urinary elimination of CML was not increased significantly when the MRP-high diet was consumed compared to consumption of the MRP-low diet, and was not proportional to the dietary exposure of CML. In conclusion it was shown that CML absorption and faecal excretion were highly influenced by dietary CML levels. Since the compound has long-term effects on health, an excessive intake deserves attention, especially in a population nutritionally at risk as adolescents.

Keywords Maillard reaction products · Adolescence · Intake · Urinary excretion · Faecal elimination · Carboxymethyllysine

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Introduction

Over the past decades food habits among adolescents have changed significantly. The tendency to eat away from home, particularly at fast-food restaurants, has increased. The frequency of snacking has risen as well and according to several studies the in-between-meals provide about one-fifth of the total energy intake but only about one-sixth of the nutrient requirements (Kun et al. 2001; Pérez Llamas et al. 2005).

Thermal treatment of foods results in the production of highly aromatic and tasty compounds, called Maillard reaction products (MRP), formed by nonenzymatic reaction of reactive sugars and proteins or peroxidised lipids, of which only a few have been defined (Sebekova et al. 2005). The formation of these products depends directly on the temperature and time of processing; and is greatly heightened by long exposure to high heat (Ames 1998; Delgado-Andrade et al. 2010). These conditions can especially affect snacks and fast foods since they are usually prepared by processes such as frying, roasting, grilling, baking and even reheated before being consumed. Thus, the MRP content in foods is related not only to their composition but

also to the method and conditions of preparation as well as with reheating (Li et al. 1994). They are widely consumed as part of the human diet (Koschinsky et al. 1997), and especially during adolescence due to their particular food pattern (Delgado-Andrade et al. 2007) and to the fact that adolescents are the most usual consumers of snacks and fast food, increasing their dietary exposure of MRP.

The information concerning the bioavailability of these newly formed species is really scarce. It has been established that about 10–30% of ingested MRP are absorbed, mainly the low-molecular fractions (Faist and Erbersdobler 2001), but very little is known about their metabolism. Most studies have been developed in rats using model systems that have exaggerated processed conditions in order to amplify the significant effects. Thus, after administration of doses of 110 or 310 mg per kg body weight of the AGE carboxymethyllysine (CML), in the form of heated casein to rats, animals eliminated in urine a 26% and a 29% of the dose and in faeces about 15 and 22%, respectively. Between 1.5–1.7% was accumulated in the circulation, liver, and kidney (Faist et al. 2000). Other studies have shown urinary excretion of CML in the range of 4–19% of that which has been ingested (Liardon et al. 1987). It has to be mentioned that there are very few human assays and those that exist are usually not consistent with data obtained in rats. Among the few that there are, most are focused on the effects of the ingestion of a concrete compound generated in conditions that are not very common in a diet. It has been well-established that complex foods used in daily life are more heavily modified than the single-protein preparations used in many studies (He et al. 1999; Van Nguyen 2006). Moreover, the MRP absorption and metabolism seem to depend on the individual chemical structure and the way they are bound to proteins (Somoza et al. 2006).

Thus, the deleterious effects of dietary MRP on human health are still unclear, except that it is well-known that MRP intake promotes oxidative stress and contributes to diabetes and the evolution of atherosclerosis and other age-related diseases (Sebekova and Somoza 2007). For this reason studies on MRP absorption and metabolism with realistic designs and concentrations of the compounds are a necessary step to establish the effects of their consumption, and its relation with the endogenously formed Maillard compounds, also called advanced glycation end-products (AGEs).

The purpose of the present study was to investigate the dietary exposure to CML as one of the most biologically active MRP and its faecal and urinary excretion in a sample of healthy male adolescents consuming the kind of a MRP-high diet, which is usually ingested by this section of the population, compared with the consumption of a MRP-low diet.

Materials and methods

Chemicals

All the chemical products and solvents for all the analyses were of the highest grade available and acquired from Sigma (Sigma-Aldrich, St. Louis, MO) and Merck (Darmstadt, Germany). CML and (D_2)-CML were provided by PolyPeptide Laboratories France SAS (Strasbourg, France).

Subjects, diets and study design

The selection of subjects, the composition of diets and the study design have been described elsewhere (Seiquer et al. 2006). Briefly, 20 male adolescents (12.4 $\pm$ 0.34 years of age, mean $\pm$ SE) participated in a 2-week randomized 2-period crossover trial, in which they consumed two different diets, with a 40-day washout period. The eating patterns of the subjects, previously evaluated in a nutritional survey, and the Recommended Intakes for the Spanish Population (Moreiras et al. 2004) were considered in the designing of the diets. The two 7-day menus had a similar content of energy and nutrients and containing the same servings per day of the different food groups. They were created as follows: MRP-low diet, free, as far as possible, of foods in which the Maillard reaction develops during cooking practices (i.e., frying, toasting, roasting) or those usually containing MRP such as bread crust or chocolate; MRP-high diet, rich in processed foods with a visible development of browning and thus rich in MRP (i.e., corn flakes, baked products, fried and breaded foods, chocolates). Lunch and dinner, the two main meals in the Spanish diet, were prepared by a local catering firm always under the strict control of the researchers (Table 1), and were distributed daily to the homes of the participants. Each of the 7-day menus was repeated twice during each 14-day experimental period. Specific instructions were given to the subjects and their parents about what to eat at breakfast and in the afternoon snack, prepared at home, for each one of the diets. The food composition of the breakfast was whole milk with sugar, white bread without crust with margarine, and fruit juice in the MRP-low diet. Whole milk with cocoa powder, breakfast cereals and fruit juice made up the breakfast in the MRP-high diet. The afternoon snack was composed of whole milk with sugar, sandwich of white bread without crust with pâté or cheese and margarine in the MRP-low diet, and whole milk with cocoa powder and pastries in the MRP-high diet.

The food composition of the diets was converted into energy and nutrient values using the Spanish Food Composition Tables (Mataix et al. 2003), under AYS44 Diet Analysis software supplied by ASDE, SA (Valencia,

Table 1 Lunch and dinner 7-days menus for the diets used for dietary treatments

DIET	1st	2nd	3rd	4th	5th	6th	7th
MRP low diet							
Lunch	Russian salad with tuna Legumes (lentils) Strawberry yoghurt Bread without crust	Spaghetti with tomato sauce, cheese and ham Bananas Bread without crust	Boiled potatoes, boiled eggs and ham Baked meat (veal) with vegetables Custard Bread without crust	Soup of pasta and chicken Sausages with mashed potatoes Bananas Bread without crust	Salad (lettuce, tomatoes…) Stewed rice Apple Bread without crust	Boiled chicken and potatoes Salad (lettuce, tomatoes…) Pears Bread without crust	Tropical salad Tuna-filled eggs Rice with milk Bread without crust
Dinner	Consommé with noodles Baked fish with boiled potatoes Pears Bread without crust	Vegetable stew Baked loin of pork and boiled potatoes Syrup peach Bread without crust	Pasta with tomatoes and cheese Bananas Bread without crust	Legumes (chickpeas) Strawberry yoghurt Bread without crust	Vegetables cream Fish pudding Custard Bread without crust	Baked fish Custard Bread without crust	Fish with cream and rice Oranges Bread without crust
MRP high diet							
Lunch	Empanadillas[a] with salad Legumes (lentils) Chocolate yoghurt Bread	Gratin macaroni with béchamel sauce Bananas Bread	Spanish omelette with ham Meatballs (veal) with vegetables Chocolate custard Bread	Legumes (chickpeas) Chocolate yoghurt Bread	Salad (lettuce, tomatoes…) Paella Apple Bread	Fried chicken and fried potatoes Salad (lettuce, tomatoes…) Pears Bread	Tropical salad Spanish omelette Rice with milk and cinnamon Bread
Dinner	Consommé with noodles Breaded fish Pears Bread	Sauté vegetables Griddle loin of pork and fried potatoes Torrija[b] Bread	Pizza Bananas Bread	Soup of pasta and chicken Hamburger with fried potatoes Bananas Bread	Vegetables cream with croutons Breaded hake fish-fingers Chocolate custard Pan	Fish croquettes Caramel custard Bread	Breaded fish and rice Oranges Bread

[a] Small tuna-filled breaded pasties

[b] Fried bread with milk, sugar and cinnamon

Spain). The overall daily content of the energy and nutrients in the study diets was as follows: energy 2,530 kcal, fat 107.5 g, carbohydrate 316.9 g, protein 90.1 g, fiber 25.1 g, cholesterol 311.4 mg, sodium 1865 mg, potassium 3826 mg, calcium 1049 mg, phosphorus 1595 mg, magnesium 372 mg, iron 17.5 mg, zinc 8.9 mg, retinol 1.4 mg, ascorbic acid 117.4 mg, α-tocopherol 11.4 mg.

To enable an analysis of the CML content in the experimental diets the catering firm also provided the meals to the researchers; both breakfast and the afternoon snack were prepared in the laboratory following the instructions given to the participants, the ingredients being purchased at a local market. The edible portions of lunch and dinner in each diet for each day (prepared in duplicate) were removed, weighed and homogenised with a hand blender (Taurus, vital CM, Spain). Aliquots from both diets over a seven-day period were well mixed and lyophilised separately, and the CML was determined. In the same manner breakfast and afternoon snacks from both diets (also in duplicate) were mixed and lyophilised to measure the CML content. The daily CML intake was calculated taken into account on the one hand, the contribution coming from the lunch and dinner and on the other the part coming from the breakfast, and the afternoon snacks.

The analysis of other Maillard reaction markers in the diets (Delgado-Andrade et al. 2007) showed a greater development of the Maillard reaction in the MRP-high diet than in the MRP-low diet. Significant higher values of hydroxymethylfurfural (HMF) and percentage of relative fluorescence intensity were measured in the MRP-high diet compared to the MRP-low diet (HMF: 0.94 ± 0.01 and 3.87 ± 0.03 mg/kg; fluorescence intensity: 7.31 ± 0.35

and 21.04 ± 0.42%, in MRP-low and MRP-high diets, respectively). No differences were found in the furosine (ε-N-(2-furoyl-methyl)-L-lysine) content between diets (6.99 ± 0.45 and 6.37 ± 0.15 mg/100 g in the MRP-low and the MRP-high diets, respectively), which confirms the conclusion that foods always contain certain amounts of early Maillard reaction compounds even when non-severe thermal treatments are applied.

The participants' compliance was assessed throughout by checking the daily record sheets in which the participants noted the details of their food consumption and recorded every food that was left over. The latter were taken into account in the calculations of CML intake. Subjects were asked to avoid restaurant and takeaway food totally during the period of dietary treatment, and to comply strictly with the recommendations.

As an approximation to the CML absorption and excretion a technique of nutrition intervention was followed. A 14-day period may be considered an adequate equilibration period that allows gastrointestinal clearance of unabsorbed CML from the previous diet (Zhi et al. 2003). At the end of each 14-day dietary treatment, a three-day collection of urine and faeces was performed (Trotter and Pohlandt 2002). 24-hour urine samples were collected on acidified containers, beginning with the second voiding of the day and finishing with the first voiding of the following day, and the volume from each daily sample was measured. The subjects were asked to report any problem with the collections, such as spillages or missed specimens. Faecal samples were weighed, and homogenized with a hand blender (Taurus, vital CM, Spain). Aliquots of urine and faeces were lyophilized and frozen at −20°C until analysis.

So far there are only few scientific publications in literature regarding the dietary exposure of MRP and metabolism from real diets. Among the most significant of these it is worth mentioning the ICARE study where different aims were proposed (Birlouez-Aragon et al. 2010). The particularity of the present study is that it was exclusively designed to evaluate the effects of MRP consumption on their metabolic fate. This is why a longer and more adequate time for the collection of urine and faeces samples was chosen to ensure a representative recovery of the CML coming from the diets.

This study was approved by the Ethics Committee of the San Cecilio University Hospital of Granada and was performed in accordance with the Helsinki Declaration of 2002, as revised in 2004. The informed consent was obtained from the parents of all the children participating in the study.

Analytical techniques

CML in the diets was analyzed previously as published in Delgado-Andrade et al. (2007). Values measured were as follows: 6.62 ± 0.25 mg/100 g of protein for the MRP-low diet and 15.72 ± 0.43 mg/100 g of protein for the MRP-low diet.

For the CML determination in urine and faeces, analyses were performed in triplicate, when possible using the method recently developed by Niquet-Léridon and Tessier (2011). Each sample was treated with sodium borohydride to stabilize the Amadori products and prevent their conversion to CML during the acid hydrolysis. A quantity of reduced sample, equivalent to 10 mg protein, was dissolved in 5 mL of 6 M HCl and incubated at 110°C for 20 h. One hundred microliters of each acid hydrolysate was dried under vacuum and reconstituted in 250 µL of internal standard containing 0.25 µg of (D_2)-CML (dissolved in NFPA 20 mM) prior to analysis by LC–MS/MS. Since this internal standard is a CML isotope it follows the same ionization as CML, increasing the precision and accuracy of the CML measurement compared with previous studies (Birlouez-Aragon et al. 2010). Liquid chromatography coupled to linear ion trap tandem mass spectrometry was used for the analysis of CML. The following instrumentation and criteria were used: Surveyor HPLC system coupled to an LTQ mass spectrometer working in its tandem operation mode (ThermoFisher Scientific, Courtaboeuf, France); thermostat, 10°C; column Hypercarb, 100 mm × 2.1 mm, 5 µm with a guard column Hypercarb, 10 mm × 2.1 mm, 5 µm; injection volume, 10 µL; flowrate, 0.2 mL/min; mobile phase, 20 mM NFPA in a water–acetonitrile gradient as follows: linear increase of acetonitrile from 0 to 50% over 20 min; ion source: electrospray ionization in positive mode; multiple reaction monitoring with the specific transitions *m/z* 205.0/130.0 and *m/z* 147.0/130.0 for CML and lysine, respectively, with a normalized collision energy of 37%.

Statistical data analysis

An SPSS for WINDOWS, version 13.0 (SPSS Inc., 1999–2004, Chicago, IL) was used for data entry and statistical analysis. The experimental data obtained after the crossover dietary treatments were analyzed by using the repeated-measures analysis of variance (ANOVA), to ascertain the consequences of the dietary treatment and to determine whether the order of presentation of the diets had an effect. There were no order effects and no treatment × order interaction for any of the dependent variables. When a significant effect between dietary treatments was found, post-hoc comparison of means was made using the Duncan test. Differences were considered significant at $P < 0.05$. The evaluation of the relationship between the different variables was carried out by computing the relevant correlation coefficient (Pearson linear correlation) at the $P < 0.05$ confidence level.

Results and discussion

As expected, based on the culinary treatments applied to MRP-low and MRP-high diets, CML intake was significantly higher ($P < 0.05$) after consumption of the latter (Table 2). This difference was also evident when body weight (BW) of subjects was taken into account to express data (197 vs. 94 μg/kgBW/day, respectively). The study of Birlouez-Aragon et al. (2010), also working with a high and a low MRP diet, showed values in the same order of magnitude as those reached in the present assay (83 and 34 μg CML/kgBW/day for the high and the low MRP diets, respectively) (Birlouez-Aragon et al. 2010).

CML faecal excretion was almost tripled after MRP-high diet intake, although when expressed as a percentage of the ingested CML no differences were found between the two experimental diets (31.7 vs. 22.5%, respectively for the high and the low-MRP diets, $P > 0.05$) (Fig. 1). Despite the increased CML faecal excretion after consumption of the MRP-high diet, the possible absorption should be higher that in the MRP-low diet, given the greater amount of CML present in the MRP-high diet. Some authors have pointed out in studies carried out in Caco-2 cells that a low rate of CML can be absorbed in the intestine by transepithelial flux, probably by simple diffusion; carriers for dipeptides and for L-lysine and L-leucine do not seem to be involved (Grunwald et al. 2006).

After the digestive process, some degraded CML could be excreted as different compounds in faeces, since it is well-established that MRP, especially Amadori compounds, are degraded by the human colonic microbiota, and thus some of the derivatives are absorbed in the intestine and some others excreted in faeces (Somoza 2005).

Table 2 CML daily intake and excretion after consumption of MRP-low and MRP-high diets in adolescent males aged 11–14 years

CML	MRP-low diet	MRP-high diet
Intake		
(mg/day)	5.36 ± 0.14[a]	11.28 ± 0.27[b]
(μg/kg/day)	94 ± 4[a]	197 ± 8[b]
Faeces		
(mg/day)	1.23 ± 0.30[a]	3.52 ± 0.52[b]
(μg/kg/day)	22 ± 6[a]	60 ± 8[b]
Urine		
(mg/day)	1.30 ± 0.14[a]	1.63 ± 0.17[a]
(μg/kg/day)	24 ± 3[a]	30 ± 3[a]

Values are means ± SE, $n = 20$. The subjects consumed the MRP-low and the MRP-high diets for 14-days periods with a 40-days washout period. Different letters in each row indicate significant differences (One way Anova and Duncan Test, $P < 0.05$)

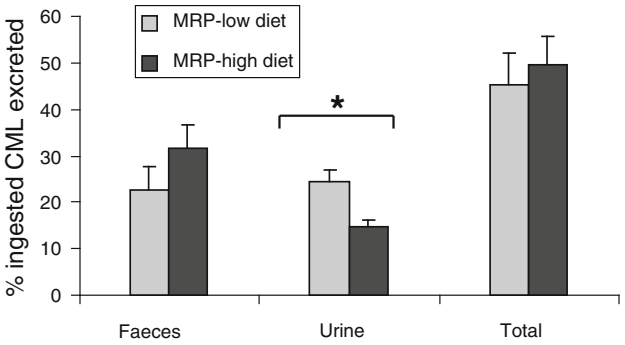

Fig. 1 Percentage of ingested CML excreted in faeces, urine and total CML excreted after consumption of MRP-low and MRP-high diets. Values are mean ± SE, $n = 20$. Significant differences between diets are depicted by a symbol (repeated measures ANOVA followed by a Duncan test, $P < 0.05$)

Once in the organism, the compound will follow a still fairly unknown metabolic transit: a fraction of the CML will become part of the circulating AGEs pool (Vlassara et al. 2002; Uribarri et al. 2003), and a portion could be deposited in target organs as liver, muscles or kidneys (Tuohy et al. 2006). Finally, another part of the compound or its metabolites will be eliminated in urine. In this line, Ahmed et al. (2004) analyzed the amount of MRP entering and leaving the liver to determine its role in the clearance of different AGEs. Apparently, this organ was not very important in the elimination of protein-bound or free MRP measured (fructoselysine, CML, N^{ε}-carboxyethyllysine, pentosidine or hydroimidazolones) in healthy subject, but instead they pointed to the kidney as the more involved organ.

In our experimental design, the urinary output was 25% higher in adolescents who consumed the MRP-high diet although the difference was not statistically significant (Table 2). When expressed as a percentage of the ingested CML, the urinary excretion was higher in the case of the MRP-low diet (Fig. 1), which would indicate that the rate of elimination of CML excreted in urine was probably limited or saturated after consumption of the MRP-high diet. The CML faecal excretion, however, did not seem to be limited and remained proportional to the intake.

Most publications have found a correlation between the intake of CML and its urinary elimination and are, therefore, in agreement with the current results. The first study to reveal this correlation was that of Liardon et al. (1987) who performed an animal study on rats. It has taken two decades to start clinical studies on the same subject. A positive but not significant association was then found by Dittrich et al. (2006) comparing infants fed with human breast milk and infant formulas. The ICARE studies confirmed its relationship for both adults and infants (Birlouez-Aragon et al. 2010).

Considering the urinary and the faecal excretion together, at the end of the crossover experimental trial, the

subjects eliminated a quantity of CML proportional to the dietary intakes. In other words not significant difference of the rate of total CML excretion was measured after consumption of the two diets (45.1 and 49.7% for the MRP-low and -high diets) (Fig. 1). This observation which is the first on healthy humans is in accordance with the animal study of Somoza et al. (2006) who fed rats with CML at two different doses for 10 days. Our percentages of CML total excretion suggest that the compound may undergo an extensive metabolism or accumulation in tissues and organs and which lead to this low recovery. There are few human trials focusing on the whole metabolic fate of different MRP or AGEs. The study of Erbersdobler and Faist (2001) evidenced that after an oral administration of fructoselysine less than 5% was found in urine or faeces. Subsequently other researchers demonstrated that the little amount detected in urine was closely dependent on dietary fructoselysine (Förster et al. 2005). In short duration clinical trials they also pointed out that almost half of the ingested pyrraline and pentosidine, other Maillard reaction derivatives, is excreted in urine (Förster et al. 2005). This same research team described only a few years before that pyrraline could be entirely released and absorbed from foods, and after its metabolic transit, eliminated rapidly and almost completely in the urine, indicating only a slight metabolization of this specific AGE within the body (Foerster and Henle 2003).

Based on the investigation of Koschinsky et al. (1997), only 10% of an oral dose of advanced MRP would be absorbed, and from that the fraction eliminated in urine would be about one-third. Although the study of Koschinsky et al. (1997) focused on total advanced MRP determined by an ELISA method, and not specifically on CML, we can attempt to compare their results with the present assay. In our experiment, the possible absorbed portion was higher than the 10% of the CML intake for both diets assayed. However, results of urinary elimination would be in agreement enough with those proposed by Koschinsky et al. (1997), especially after consumption of the MRP-low diet, where the urinary excretion represented one-third of the amount absorbed. In the case of the MRP-high diet that amount of CML excreted would be one-sixth of the absorbed one. Thus, the consumption of this diet might exceed the detoxification capacity of the body; or perhaps all the opposite, that the CML supply was so high that it forced a more rapid metabolism and clearance of its metabolites, hence the low recovery.

Therefore, as depicted in Fig. 2, much of the ingested CML has an unknown destination in the body. Once in the gastrointestinal tract, the microbial activity will degrade a portion. The rest could be absorbed or eliminated as it is in faeces. For the absorption, according to the statements of Broer (2008), a carrier-mediated transport system could be also involved. Next, a fraction will reach the systemic circulation intact and will be transported to different tissues/organs where metabolism can occur (Somoza et al. 2006). Thus, a part is excreted intact in urine and another fraction could be degraded into unidentified metabolites and also excreted in urine. As described for different MRP, during its journey through the different tissues the accumulation of CML or its metabolites has been also stated (Tuohy et al. 2006).

Some studies focusing on the metabolic fate of MRP have also analyzed the protein-bound CML levels in plasma as a marker of its distribution in the body (Birlouez-Aragon et al. 2010; Sebekova et al. 2001). The ICARE study (Birlouez-Aragon et al. 2010) indicated a significant difference, but an extremely low one, between the protein-bound CML in the plasma of the subjects consuming a high or low MRP diet. For that reason there is a growing interest in the measurement of the free CML levels in plasma as a real index of the CML fraction coming from the diet, and which is partly excreted in the urine. In the present study the free CML in plasma was intended to be measured using the LC–MS/MS method described; however it was not possible due to methodological difficulties.

Another aim of this study was to search for correlations between CML and nitrogen intakes, and CML and nitrogen elimination knowing that diets rich in MRP have a negative effect on protein digestibility (Seiquer et al. 2006). The correlations were calculated using the Pearson linear correlation coefficient (Table 3). CML and nitrogen intakes were positively correlated only in the case of the MRP-low diet. The correlation was lost in volunteers who took the MRP-high diet. To understand this fact we must keep in mind that both diets were designed with the same protein content. The extension of protein browning due to the culinary treatment applied was the only different between them. Thus, subjects consuming the MRP-high diet had a higher CML intake than those fed the MRP-low diet, but eating the same amount of protein. Probably for this reason the proportionally between CML and nitrogen intake was lost in this diet.

A statistically significant correlation between the CML and nitrogen faecal excretions was found when all the periods were analyzed. However when the two diets were analyzed separately the correlation was maintained only in the MRP-high diet. This accords with the results obtained previously from our experimental group which showed that the digestibility of nitrogen was reduced in those participants consuming the MRP-high diet (Seiquer et al. 2006). It is in fact well-established that MRP consumption decreases not only the digestibility of nitrogen which is damaged but also that which is intact (Mougham et al. 1996). In the case of the MRP-low diet the loss of the correlation could be to some extent explained as a consequence of a different enzymatic action during the digestive

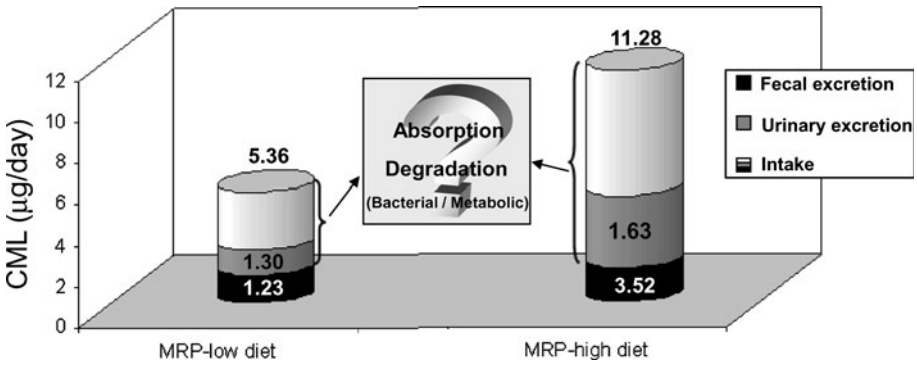

Fig. 2 Diagram of metabolic fate of CML after consumption of MRP-low and MRP-high diets

Table 3 Correlations established between intake, faecal and urinary excretion of CML and nitrogen after consumption of MRP-low and MRP-high diets

Periods	Nitrogen-CML intake	Nitrogen-CML faecal excretion	Nitrogen-CML urinary excretion
The whole assay	$P > 0.05$	$r = 0.6345$ $P = 0.0002$	$P > 0.05$
MRP-low diet period	$r = 0.5800$ $P = 0.0185$	$P > 0.05$	$r = 0.5994$ $P = 0.0141$
MRP-high diet period	$P > 0.05$	$r = 0.6430$ $P = 0.0131$	$P > 0.05$

process of both diets. It has been long established that MRP affect the enzymatic attack of proteins due to the appearance of steric impediments or even to the inhibition of proteases (O'Brien and Morrissey 1989; Pitotti et al. 1994). It could therefore be hypothesised that in the low-MRP diet the CML linked to protein was easily released and the nitrogen was available to be absorbed, while in the MRP-high diet a higher amount of CML could remain bound to the protein backbone and limit the enzymatic action, leading to a combined excretion of nitrogen and CML.

For the urinary excretion it must be underlined that due to the metabolic transit of the compound it is quite difficult to present a solid hypothesis to explain the correlations found. A relationship between nitrogen and CML elimination appeared only in the MRP-low diet. The fact that no such correlation exist in the results obtained from those consuming the MRP-high diet could be related to the higher CML absorbed there. This leads to the supposition that there is an increased rate of metabolization with that compound, and a possible excretion of unidentified metabolites in urine.

Conclusions

Data of the present study are in line with other available in the literature and let us confirm that CML absorption and faecal excretion is highly influenced by dietary CML levels. The absorption mechanism, the metabolic transit and the target organs for accumulation of CML will necessitate further investigation for a full understanding of its metabolic transit in vivo as well as its health effects. The implication of CML in pathogenesis and development of different degenerative disorders might be taken into account when a MRP rich diet is consumed, situation becoming more frequent due to the modification of dietary habits of adolescents, but also of the general population in Western countries.

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