

A casein variant in cow's milk is atherogenic

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Abstract

Casein is a major protein in cow's milk that occurs in several variant forms, two of which are β -casein A¹ and β -casein A². The levels of these two proteins vary considerably in milk dependent on the breed of cow, and epidemiology studies suggest that there is a relationship between their consumption and the degree of atherosclerosis. In the present study, the direct effect of consumption of β -casein A¹ vs β -casein A² on atherosclerosis development was examined in a rabbit model. Sixty rabbits had their right carotid artery balloon de-endothelialised at $t = 0$, divided randomly into 10 groups ($n = 6$ per group), then for 6 weeks fed a diet containing 0, 5, 10 or 20% casein isolate, either β -casein variant A¹ or A², made up to 20% milk protein with whey. Some groups had their diets supplemented with 0.5% cholesterol. Blood samples were collected at $t = 0, 3$ and 6 weeks and rabbits were sacrificed at $t = 6$ weeks. In the absence of dietary cholesterol, β -casein A¹ produced significantly higher ($P < 0.05$) serum cholesterol, LDL, HDL and triglyceride levels than whey diet alone, which in turn produced higher levels than β -casein A². Rabbits fed β -casein A¹ had a higher percent surface area of aorta covered by fatty streaks than those fed β -casein A² (5.2 ± 0.81 vs 1.1 ± 0.39 , $P < 0.05$) and the thickness of the fatty streak lesions in the aortic arch was significantly higher (0.04 ± 0.010 vs 0.00 , $P < 0.05$). Similarly, the intima to media ratio (I:M) of the balloon injured carotid arteries in A¹ fed animals (0.77 ± 0.07) was higher than in those that consumed A² (0.57 ± 0.04) or whey (0.58 ± 0.04), but this did not reach significance. In the presence of 0.5% dietary cholesterol, the thickness of the aortic arch lesions was higher ($P < 0.05$) in 5, 10 and 20% casein A¹ fed animals compared with their A² counterparts, while other parameters were not significantly different. It is concluded that β -casein A¹ is atherogenic compared with β -casein A².

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1. Introduction

Cow milk constitutes a major part of the diet of developed nations and it contains an average 32 g of protein per litre [1], mostly casein (82%) and whey (18%). The caseins have been divided into four subclasses, α_{s1} , α_{s2} , β and κ [2,3], whose major function is to transport calcium phosphate [4]. β -Casein is the second most abundant casein behind α_{s1} [1] and is essential for curd formation and important in determining the surface properties of micelles [4]. To date 10 genetic variants of β -casein have been characterised with regard to sequence [5,6], and of these only A¹, A², A³ and B are found in nearly all *Bos taurus* populations [7,8]. The allelic distribution of variants is such that the majority

of cows in Western nations produce milk rich in the genetic variant β -casein A¹. For example, Holstein and Ayrshire breeds of cow produce 63 and 67% A¹, respectively, and 35 and 33% A² [9]. In contrast, the Guernsey breed produces 1% A¹ and 98% A². Alignment of β -casein A¹ and β -casein A² protein sequences reveals near-perfect homology with the only difference at codon 67 where β -casein A² has a proline residue and β -casein A¹ has a histidine residue [4,10].

While some epidemiological studies show no association between reported milk consumption and cardiovascular mortality [11], comparative data from several populations have suggested a strong correlation between the consumption of β -casein A¹ and deaths from ischaemic heart disease. McLachlan [12] examined the calculated consumption of β -casein A¹ (excluding cheese) against IHD mortality in males aged 30–69 in 16 developed nations and found a correlation value (r^2) of 0.86. Upon comparing IHD and β -casein A¹ con-

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sumption in the states of the former West Germany, the same researcher found an $r^2 = 0.66$, three times greater than the correlation between smoking and IHD in the same population. This implies that β -casein A¹ consumption may cause more vascular damage than a well-known risk factor for atherosclerosis.

There have been almost no cases of IHD-related mortality reported in either the Masai (East Africa) [13] or Samburu (Northern Kenya) [14] communities. Furthermore, these tribes showed normal to low cholesterol levels despite having a diet rich in animal milk and meat protein [13,14]. Interestingly, almost all of the African Zebu (*B. indicus*) cattle populations produce milk containing β -casein A² [15].

Ingestion of total casein, the protein fraction isolated by acid precipitation of all casein subclasses from milk, has been shown to promote atherosclerosis in several animal models including rabbits [16], monkeys [17] and mice [18]. Casein consumption is also associated with hypercholesterolemia [19] and reduced LDL catabolism [20]. However, to date no controlled diet studies have been carried out to definitively determine whether a specific casein subclass or subclass variant is more atherogenic than others. The aim of the present study was to determine whether dietary administration of β -casein A¹ in a rabbit model of atherosclerosis promotes the disease state compared with rabbits fed β -casein A².

2. Materials and methods

2.1. Study groups

Sixty New Zealand white/Lop cross rabbits of both sexes (16–24 weeks old) had their right carotid artery balloon de-endothelialised at $t = 0$ [21], then randomly divided into 10 groups ($n = 6$ per group). Group 1 was fed pellets containing 20% whey protein for 6 weeks while Groups 2 and 3 had 10% β -casein A¹ and A², respectively, plus 10% whey, all with no added dietary cholesterol. The diets for the remaining seven groups contained 0.5% dietary cholesterol and 20% milk protein, which differed only in the proportions of whey and casein. Group 4 had 20% whey protein while Groups 5 and 6 received diets containing 10% β -casein A¹ and A², respectively, plus 10% whey. Groups 7 and 8 had 5% β -casein A¹ and A², respectively, plus 15% whey protein, while Groups 9 and 10 received 20% β -casein A¹ and A², respectively. All diet pellets were specially prepared by Dyets, Inc., Philadelphia, PA.

In a pilot study, it was found that it took a few days for the rabbits to become accustomed to their new diets that differed from the normal pellets in colour, smell, texture and weight. Most obviously, the study pellets were white while normal pellets were brown. Thus, for 5 days prior to surgery, all rabbits were weaned onto their

respective diets by mixing increasing amounts of study pellets (25, 50, 75 and 100%) with normal rabbit pellets. At $t = 0$, only study pellets were offered for the following 6 weeks and water was available ad libitum. The amount of pellets eaten by each rabbit was determined each day. All rabbits were weighed weekly, commencing the day of surgery until the week of sacrifice. Blood was collected from each rabbit prior to surgery, at the commencement of diet and at $t = 3$ and 6 weeks. The serum supernatant was extracted and analysed at Queensland Medical Laboratories, Brisbane, for total serum cholesterol, triglycerides, HDL, LDL and homocysteine.

At $t = 6$ weeks, all animals were euthanased by an overdose of pentobarbitone (325 mg/ml Lethobarb, Virbac Australia Pvt. Ltd., Vic.), then immediately perfused fixed with 4% formaldehyde (Asia Pacific Specialty Chemicals Ltd., NSW, Australia) in phosphate-buffered saline at 100 mmHg. The entire aorta, from the iliac bifurcation to the aortic arch, was exposed and removed. The right (injured) and left (control) common carotid arteries were fully exposed and removed.

2.2. *En face* Oil-Red-O staining for fatty streak lesions

The thoracic and abdominal aorta minus the aortic arch (approximately 16 cm) was opened longitudinally and cut into four segments. Each segment was sewn (lumen side up) onto small pieces of clear plastic to ensure they remained flat. The segments were then stained *en face* with a saturated solution of the lipophilic dye Oil-Red-O.

Images of each aortic segment were scanned using Desk Scan II (version 2.31a, Hewlett-Packard, USA) and both the lesion and total area measured using the ImageJ imaging software (version 1.23y, National Institutes of Health, USA). The results were expressed as the percentage of luminal surface area covered by fatty streak lesions.

2.3. *Histological sections/intima to media ratio*

Three segments of 3–4 mm length were cut from each aortic arch, dehydrated, embedded in plastic, sectioned and stained with Toluidine Blue. Similarly, four or five segments each of 3–4 mm in length were cut from the same regions spanning the full length of the left (control) and the right (ballooned) carotid arteries, respectively, and prepared in the same manner.

All morphometric analyses of the intima to media ratios (I:M) of the ballooned and control carotid arteries and the aortic arch were performed using the Mocha Image Analysis system (version 1.1, Jandel Scientific, CA). Measurements were taken to determine the size (in pixels) of both the intima and media layers in all

segments of each artery. The ratio of intima to media was calculated and recorded for each of the three aortic arch segments per rabbit, the four control carotid segments and the five ballooned carotid segments.

2.4. Statistics

All statistical analyses were performed using the SigmaStat (Jandel Scientific, CA) statistical software package. Comparison of data from the morphometric analyses were carried out with one-way multiple ANOVAs, using the Kruskal–Wallis analysis of variance on ranks test. In all statistical analyses, a *P*-value of less than 0.05 was considered significant. All data have been reported as mean \pm standard error of mean.

3. Results

3.1. Rabbit diets and weights

While the amount of the study pellets eaten each day varied between rabbits, on group averages the amounts consumed varied by only 12 g per day over the 6 weeks experimental period, and there was no significant difference ($P < 0.05$) between any of the groups (mean = 30.05 ± 0.9 g per day). The addition of 0.5% cholesterol to the diets of Groups 4–10 did not influence the amount eaten, or did the type or amount of casein. Almost all rabbits lost weight over the 6 weeks of study (mean -5.62%) with no significant difference between groups ($P < 0.05$).

3.2. Serum cholesterol and LDL/HDL ratio

At week 6, Group 3 (A^2) serum cholesterol was significantly lower than all groups including Groups 1 (10% whey) and 2 (A^2) (Fig. 1A). Interestingly, Group 2 (A^1) was higher than Group 1, but this was not significant. Of note, the serum cholesterol level in Group 10 (20% A^2 plus 0.5% dietary cholesterol) was approximately half that of Group 4 (20% whey plus 0.5% dietary cholesterol) and Group 9 (20% A^1 plus 0.5% dietary cholesterol) (12.1 ± 1.9 mmol/l vs 24.7 ± 5.7 and 22.4 ± 6.8 mmol/l, respectively) (see Fig. 1A and Table 1).

Group 3 (A^2) LDL levels were significantly lower than Groups 1 and 2. All groups with added dietary cholesterol (Groups 4–10) were not significantly different from each other. These results, however, may have been swamped by the relatively high level of dietary cholesterol (0.5%), whereas a lower cholesterol load may have shown more striking differences. Serum HDL followed the same pattern as serum LDL. HDL in Group 2 (A^1) was significantly higher than Group 3 (A^2) but not significantly different from Group 1. The LDL

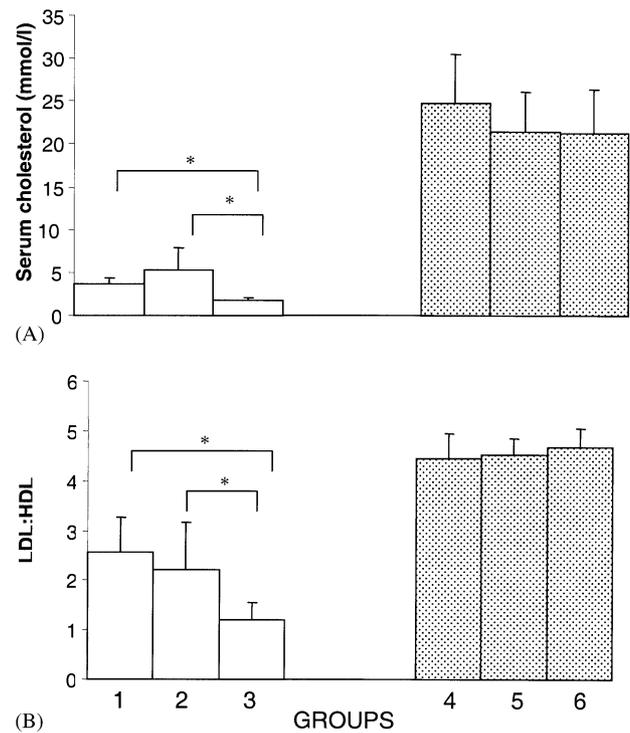


Fig. 1. (A) Total serum cholesterol levels (mmol/l) of rabbit Groups 1–6 at week 6. (B) Serum LDL to HDL ratio at week 6. Group 1: 20% whey diet; Group 2: 10% β -casein A^1 +10% whey; Group 3: 10% β -casein A^2 +10% whey; Group 4: 20% whey+0.5% cholesterol; Group 5: 10% β -casein A^1 +0.5% cholesterol; and Group 6: 10% β -casein A^2 +0.5% cholesterol. * $P < 0.05$.

to HDL ratio of Group 3 (A^2) was significantly lower than all other groups including Group 1 (20% whey) and Group 2 (10% β -casein A^1), while the LDL to HDL ratio of all groups with dietary cholesterol (Groups 4–10) were not significantly different from each other (see Fig. 1B). However, there was a trend for higher LDL to HDL ratios with increasing doses of β -casein A^1 with dietary cholesterol (4.19 ± 0.57 , 4.51 ± 0.34 and 4.99 ± 0.67) and lower levels with increasing doses of β -casein A^2 with dietary cholesterol (5.63 ± 1.75 , 4.68 ± 0.37 and 4.66 ± 0.25), but this was not significant (see Fig. 1B, Table 1).

3.3. Triglycerides and homocysteine

By week 6, the serum triglyceride levels for most groups had fallen from those at week 0, and showed no significant difference between groups. Serum homocysteine levels changed only slightly from those at week 0, with no significant differences between the groups (data not shown).

Table 1

Data from rabbit Groups 7–10 at week 6 showing total serum cholesterol, serum LDL to HDL ratio, percent luminal surface area of aorta covered by lipid-filled lesions, thickness of lesions in aortic arch expressed as intima to media ratio (I:M) and thickness of lesions in injured carotid artery expressed as intima to media ratio (I:M)

Group	Chol (mmol/l)	LDL/HDL	Lesion (%)	Aorta I:M	Carotid I:M
7	18.6±3.7	4.19±0.57	10.6±1.54	0.04±0.01*	0.75±0.08
8	18.4±5.5	5.63±1.75	5.2±1.04	0.00±0.00*	0.70±0.08
9	22.4±6.8	4.99±0.67	6.4±1.57	0.05±0.01**	0.78±0.08
10	12.1±1.9	4.66±0.25	2.2±0.48	0.00±0.00**	0.65±0.05

Group 7: 5% β -casein A¹+15% whey+0.5% cholesterol; Group 8: 5% β -casein A²+15% whey+0.5% cholesterol; Group 9: 20% β -casein A¹+0.5% cholesterol; and Group 10: 20% β -casein A²+0.5% cholesterol.

* $P < 0.05$.

** $P < 0.05$.

3.4. Aortic fatty streaks

Group 1 rabbits fed 20% whey had 0% aortic luminal surface area covered by plaque (Fig. 2A). Group 3 rabbits (10% β -casein A² plus 10% whey) had only 1.1±0.4% surface area covered by plaque, and this was not significantly higher than Group 1. In contrast, the plaque surface area of Group 2 rabbits fed 10% β -casein A¹ plus 10% whey was 5.2±0.8%. All animals given a 0.5% cholesterol-enriched diet had values that were not significantly different from each other, irrespective of casein variant, although 20% β -casein A² with dietary cholesterol produced lesions covering only 2.2±0.48% of the aortic luminal surface compared with 6.4±1.57% for 20% β -casein A¹ under the same conditions (see Table 1). As with the other parameters measured, the addition of a dietary cholesterol load less than 0.5% may have produced more striking results. Interestingly, 10% β -casein A¹ without dietary cholesterol (Group 2) produced about the same degree of plaque as 10% β -casein A¹ or A² with dietary cholesterol (Groups 5 and 6) (see Fig. 2A). When the thoracic and abdominal segments of aorta were analysed separately, the same overall results were obtained.

When histological sections of the aortic arch were analysed morphometrically, there was no measurable intimal thickening in the presence of 20% whey alone (Group 1) or 10% β -casein A² with no dietary cholesterol (Group 3) (Fig. 2B). The intima to media ratio in rabbits fed 10% β -casein A¹ in the absence of dietary cholesterol (Group 2) was significantly higher than both of these groups, and produced almost the same degree of intimal thickening as 20% whey with 0.5% dietary cholesterol (Group 4) (0.04±0.01 vs 0.03±0.01, $P < 0.05$). Of note, in the presence of dietary cholesterol, the intima to media ratio in the aortae of Group 7 rabbits (5% β -casein A¹) was significantly higher than in Group 8 (5% β -casein A²) (Table 1). Also, the value in Group 9 rabbits (20% β -casein A¹) was significantly higher than in rabbits fed 20% β -casein A² (Group 10). Groups 8 and 10 (5 and 20% β -casein A² plus dietary

cholesterol) had no measurable intimal thickening, while Group 6 (10% A² β -casein with dietary cholesterol) had a mean intima to media ratio of only 0.01, with four out of the six rabbits in this group having 0%. All β -casein A¹ groups had higher intima to media ratios than groups fed β -casein A² ($P < 0.05$) and had intima to media ratios not significantly different from Group 4 (20% whey with 0.5% cholesterol).

3.5. Advanced lesions in carotid arteries

Balloon catheter injury to the right carotid artery at $t = 0$ induced a hyperplastic neointimal thickening in all rabbits by 6 weeks (Fig. 2C). Measurement of intima to media ratios showed that in the absence of dietary cholesterol there was a slight, but not significant, increase in neointimal thickening in Group 2 rabbits (10% β -casein A¹) compared with Group 3 (10% β -casein A²) and Group 1 (20% whey). Likewise, the groups fed 5, 10 and 20% β -casein A¹ plus 0.5% cholesterol had thicker neointimas than Group 1. Groups fed 5, 10 and 20% β -casein A² plus 0.5% cholesterol had thinner neointimas than their β -casein A¹ counterparts (see Fig. 2C, Table 1).

There was no neointimal thickening observed in any of the contralateral uninjured carotid arteries.

4. Discussion

When rabbits are fed a cholesterol-enriched diet, subendothelial accumulations of fat-filled macrophages (“foam cells”) are developed in most arteries [22]. These lesions are termed fatty streaks and closely resemble human juvenile fatty streaks that are present in early childhood and are considered the precursors of advanced atherosclerotic plaques [23]. Since juvenile fatty streaks are first seen in infants under 3 years of age [23], the question was raised: is casein, in addition to milk fat, responsible for fatty streak formation? Casein (as an unspecified mixture of variants) has been shown to

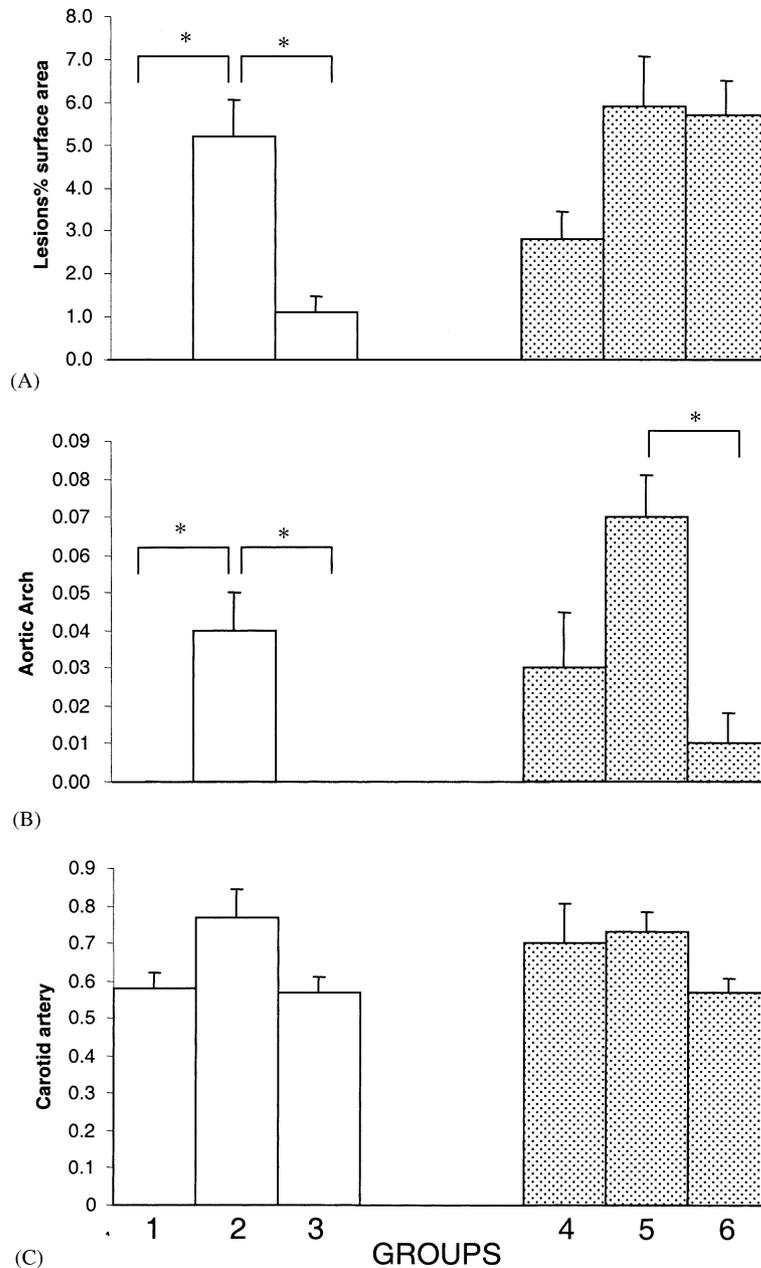


Fig. 2. (A) Percent luminal surface area of aorta covered by lipid-filled lesions. Note that in Group 1 there were no areas of lipophilic staining. (B) Thickness of lesions in aortic arch expressed as intima to media ratio (I:M). Note that there was no intimal thickening in Groups 1 and 3. (C) Thickness of lesions in balloon injured carotid artery expressed as I:M. Group 1: 20% whey diet; Group 2: 10% β -casein A¹+10% whey; Group 3: 10% β -casein A²+10% whey; Group 4: 20% whey+0.5% cholesterol; Group 5: 10% β -casein A¹+0.5% cholesterol; and Group 6: 10% β -casein A²+0.5% cholesterol. * $P < 0.05$.

promote atherosclerosis in several animal models, including rabbits [16,24], monkeys [17] and mice [18]. None of these studies, however, defined which particular casein variant was responsible.

In the present study, using a rabbit model it was found that both β -casein A¹ and A² led to aortic fatty streaks but A¹ produced considerably more extensive lesions than both A² and whey. Indeed, 10% A¹ without dietary cholesterol produced about the same luminal surface area of the aorta covered by lesions as 10% A²

with added dietary cholesterol. The thickness of fatty streaks in the aortic arch of rabbits fed A¹ was significantly greater than that in rabbits fed A² or whey. Even in the presence of 0.5% dietary cholesterol, the thickness of these lesions was significantly greater in all A¹ than A² groups. Thus A¹ is more atherogenic than both the whey and the A² casein variant.

A secondary aspect of this study was to investigate the potential of β -casein A² to exert an athero-protective effect compared with whey in the presence of 0.5%

dietary cholesterol. Only 20% A² produced a smaller surface area of aorta covered by fatty streaks than 20% whey under these conditions. However, aortic arch fatty streak thickness was significantly smaller for both 20 and 5% A² fed animals compared with 20% whey. These results, combined with the fact that 10% A² with no added cholesterol produced a significantly lower serum cholesterol level and LDL to HDL ratio than all other groups including whey only and 10% A¹, demonstrate for the first time that β -casein A² has a mildly athero-protective effect while β -casein A¹ is most definitely atherogenic.

These results are consistent with epidemiological studies that suggest a strong relationship between mortality from cardiovascular disease and consumption of β -casein A¹, even though other studies show no association between reported milk consumption per se and cardiovascular mortality [11]. In the states of the former West Germany, where allelic composition of regional cattle herds has remained virtually constant since 1950s, IHD mortality by state correlates with the estimated consumption of the β -casein A¹ allele [12]. Also, the IHD mortality rates of the populations of Toulouse in France and Belfast in northern Ireland vary more than threefold, despite having nearly identical traditional risk factors for heart disease. Interestingly, people from Belfast are estimated to consume 3.23 times more β -casein A¹ than those from Toulouse. The communities recorded as being essentially free from IHD but also drink milk, the Masai [13] and Samburu [14] of Africa, obtain their milk from Zebu cattle, which produce only the β -casein A² allele and no β -casein A¹ [15].

What are the possible mechanisms by which the different casein variants affect the development of atherosclerosis? Firstly, there is the effect of β -casein A² on lowering serum cholesterol. Secondly, β -casein A¹, but not β -casein A², releases β -casomorphin-7 (β -CM-7) [25], which is thought to be involved in the oxidation of LDL through a peroxidase-dependent process. β -Casomorphins have been detected in the plasma of newborn calves [26] and in the small intestines of humans after cow milk consumption [27]. Pancreatic elastase releases the carboxyl terminus of β -CM-7 by degrading the peptide bond between Ile⁶⁶ and His⁶⁷ of β -casein A¹. However, elastase is unable to cleave the bond between Ile⁶⁶ and Pro⁶⁷ of β -casein A², and so β -CM-7 cannot be released. Analysis of protein oxidation products isolated in atherosclerotic lesions has implicated the tyrosyl radical (among others) in LDL oxidation [28]. Torreilles and Guerin [29] found that bovine casein-derived peptides with tyrosyl-end residues (such as β -CM-7) could promote peroxidase-dependent oxidation of human LDL, implying that the tyrosyl ending peptide is a diffusible catalyst that conveys oxidising potential to LDL lipids. Finally, the fact that

there was no difference in intima to media ratio in the balloon injured right carotid artery of rabbits fed A¹, A² or whey may point to possible mechanistic differences. Endothelium was present in the aorta but absent in the right carotid artery, and this suggests different effects on the endothelium following ingestion of the two β -caseins, perhaps in relation to expression of adhesion molecules such as ICAM-1 which in turn leads to monocyte/macrophage invasion of the vessel wall [30,31].

What might the differential effects of the casein variants have on the wider community? β -Casein allele frequency data indicate that of the four major milk-producing breeds of cow in the Western world, half produce milk that is rich in β -casein A¹ and only one of the four breeds produces milk that is almost purely A². This means that the milk sold in supermarkets and other stores will contain a high proportion of the β -casein A¹ allele. Since children develop juvenile fatty streaks as a precursor to more advanced lesions in later life, the type of casein ingested in childhood may be critical to the likelihood of developing atherosclerosis. The public could be offered the healthier choice of pure A² milk, which would be particularly important for the children of persons with known cardiovascular disease. Since the milk from Guernsey cows contains 98% A², it may be possible to change herd allelic composition by 'breeding out' those cows with A¹/A¹ alleles. These measures may thus contribute to improve human health by reducing a risk factor that contributes to ischaemic heart disease, the leading cause of premature death in the developed world today.

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