Study on the Preventive Physiological Functions of the Casein-Derived Tripeptide, Met-Lys-Pro, Against Hypertension and Dementia

湯田, 直樹

Study on the Preventive Physiological Functions of the Casein-Derived Tripeptide, Met-Lys-Pro, Against Hypertension and Dementia

Naoki Yuda

2021

Acknowledgements

I would like to express my deepest gratitude to Professor Takuya Kitaoka at Kyushu University for his generous support, suggestions, and discussion on this doctoral dissertation. I would also like to express my sincere thanks to Professor Toshiro Matsui, Professor Yoshinori Katakura, and Associate Professor Hirofumi Ichinose at Kyushu University for their valuable suggestions and discussion in carefully reviewing this doctoral dissertation.

I would like to express my appreciation to Dr. Naoki Sakane at National Hospital Organization Kyoto Medical Center for his insightful comments and thoughtful guidance on the clinical trials.

I would like to express my gratitude to Dr. Masahiko Nakamura at Matsumoto City Hospital for his generous support and constructive comments on the clinical trial of cognitive function. I am also greatly indebted to Professor Mitsunaga Miyasaka, Ms. Izumi Kakiuchi, and Ms. Kyoko Kiyosawa at Matsumoto Junior College for their enormous support and encouragement in conducting the clinical trial. I would like to thank Matsumoto City and Matsumoto Health Lab staff for their great contribution to the clinical trial, too.

I am grateful to Dr. Masahiko Tokushima at Maebashi North Hospital for his considerable support and contribution to the clinical trial of safety evaluation.

I would like to show my appreciation to Mr. Michio Miyahara, Chairman of Morinaga Milk Industry Co., Ltd., Mr. Yoichi Onuki, President of Morinaga Milk Industry Co., Ltd., and Dr. Teiichiro Okawa, Executive Vice President of Morinaga Milk Industry Co., Ltd. for giving me the opportunity to conduct this study.

I would like to offer my special thanks to Dr. Fumiaki Abe and Dr. Miyuki Tanaka at Morinaga Milk Industry Co., Ltd. for their incisive comments and tremendous support. I am also thankful to Dr. Koji Yamauchi, Dr. Hiroshi Ochi, Mr. Akio Yamada, Mr. Manabu Suzuki, and Mr. Daisuke Ochi at Morinaga Milk Industry Co., Ltd. for their valuable suggestions and huge help. I am highly indebted to all my colleagues at Morinaga Milk Industry Co., Ltd. for their kind assistance. I am also grateful to Dr. Keiji Iwatsuki and Dr. Yuzo Asano for their constructive comments and warm encouragement.

I would particularly like to thank Professor Hiroyuki Wariishi and all the members of the Laboratory of Bioresources Chemistry at Kyushu University for teaching me how to enjoy research.

Last but not least, I would like to express my sincere appreciation to my wife Yumi, my daughters Haruko and Mahiro, my mother Mihoko, my father Toru, and my entire family for their generous support.

Naoki Yuda

September 2021

Abbreviations

ACE	Angiotensin I-Converting Enzyme
AD	Alzheimer's Disease
ADAS-cog	Alzheimer's Disease Assessment Scale-cognitive subscale
AE	Adverse Event
AT1R	Angiotensin II Type 1 Receptor
Ang (1–7)	Angiotensin 1–7
Ang (1–9)	Angiotensin 1–9
Ang I	Angiotensin I
Ang II	Angiotensin II
Ang III	Angiotensin 2–8
Ang IV	Angiotensin 3–8
Aβ	Amyloid-Beta
baPWV	Brachial-Ankle Pulse Wave Velocity
BMI	Body Mass Index
BP	Blood Pressure
BW	Body Weight
CAA	Consumer Affairs Agency
CK	Creatine Kinase
CTCAE	Common Terminology Criteria for Adverse Events
CVD	Cardiovascular Disease
DBP	Diastolic Blood Pressure
EC	(−)-Epicatechin
ECG	(−)-Epicatechin Gallate
EGC	(−)-Epigallocatechin
EGCG	(−)-Epigallocatechin Gallate
ESI	Electrospray Ionization
FFC	Foods with Function Claims
FNFC	Foods with Nutrient Function Claims
FOSHU	Foods for Specified Health Uses
GDS	Geriatric Depression Scale
HCW	Hot-Compressed Water
HDS-R	Revised Version of Hasegawa's Dementia Scale
HMF	5-Hydroxymethyl-2-Furaldehyde
HPLC	High-Performance Liquid Chromatography

HbA1c	Hemoglobin A1c
IC_{50}	Half Maximal Inhibitory Concentration
ICV	Intracerebroventricular
IPP	Ile-Pro-Pro
JPY	Japanese Yen
LC-MS	Liquid Chromatography-Mass Spectrometry
MCH	Mean Corpuscular Hemoglobin
MCI	Mild Cognitive Impairment
MCS	Mental Health Component Summary
MCV	Mean Corpuscular Volume
MKP	Met-Lys-Pro
MoCA-J	Japanese Version of the Montreal Cognitive Assessment
OADR	Old-Age Dependency Ratio
OBR	Occult Blood Reaction
PCS	Physical Health Component Summary
RAS	Renin-Angiotensin System
RCT	Randomized Controlled Trial
SBP	Systolic Blood Pressure
SD	Standard Deviation
SE	Standard Error
SF-8	Eight-Item Short-Form Health Survey
SHR	Spontaneously Hypertensive Rat
SR	Systematic Review
TF	Theaflavin
TF3,3'G	Theaflavin 3,3'-*O*-Gallate
TF3G	Theaflavin 3-*O*-Gallate
TF3'G	Theaflavin 3'-*O*-Gallate
U-bil	Urine Bilirubin
U-glu	Urine Glucose
U-ket	Urine Ketone Body
U-pH	Urine pH
U-pro	Urine Protein
U-uro	Urine Urobilinogen
UA	Uric Acid
UMIN-CTR	University Hospital Medical Information Network Clinical Trials Registry
USG	Urine Specific Gravity
VPP	Val-Pro-Pro

Table of Contents

List of Figures

List of Tables

Chapter 1

General Introduction

1.1 Progress of Global Aging

Populations are rapidly aging worldwide. Globally, there were 727.6 million persons aged 65 years and above in 2020 [1]. Projections indicate that 15.9% of the world population will be aged 65 years and above by 2050, from 9.3% in 2020 (Figure 1-1). Population aging is the result of human success through medical, public health, technologies, economics, and social development. Conversely, to support an aging society, it is important to prevent illness and to lead a healthy life. The progress of aging is expected to be remarkable in East Asia, Southeast Asia, Europe, and Central America. The old-age dependency ratio (OADR) is one of the most commonly used indexes for monitoring changes in the age structure of populations. The OADR is defined as the number of persons aged ≥ 65 years per 100 persons of working age (20 to 64 years). Figure 1-2 shows the top 10 countries or areas with the highest OADR in 2020 and by 2050. Japan is expected to have the highest aging rate in the world, not only presently but also in the future. In other words, the progress of aging is a global trend, and Japan is at the forefront. Therefore, building a healthy and prosperous aging society in Japan can be a model case for the world.

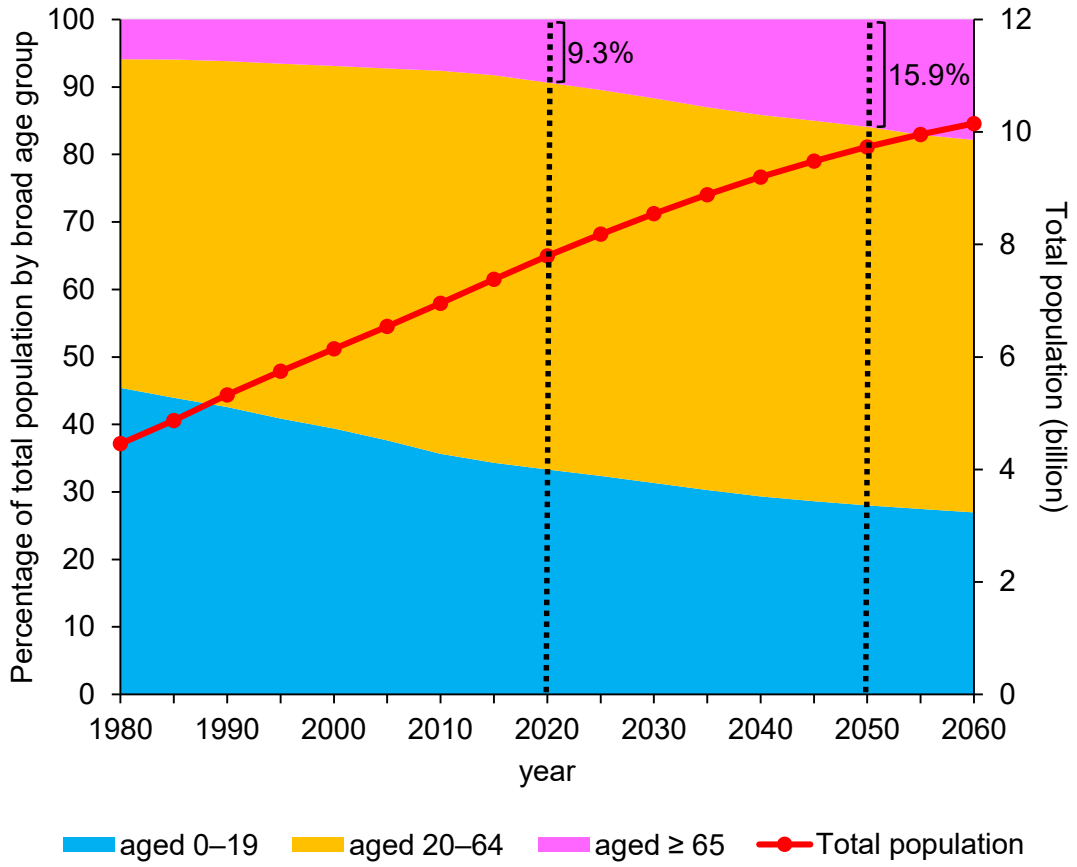

Figure 1-1 Global demographics.

Data source: United Nations, World Population Prospects 2019.

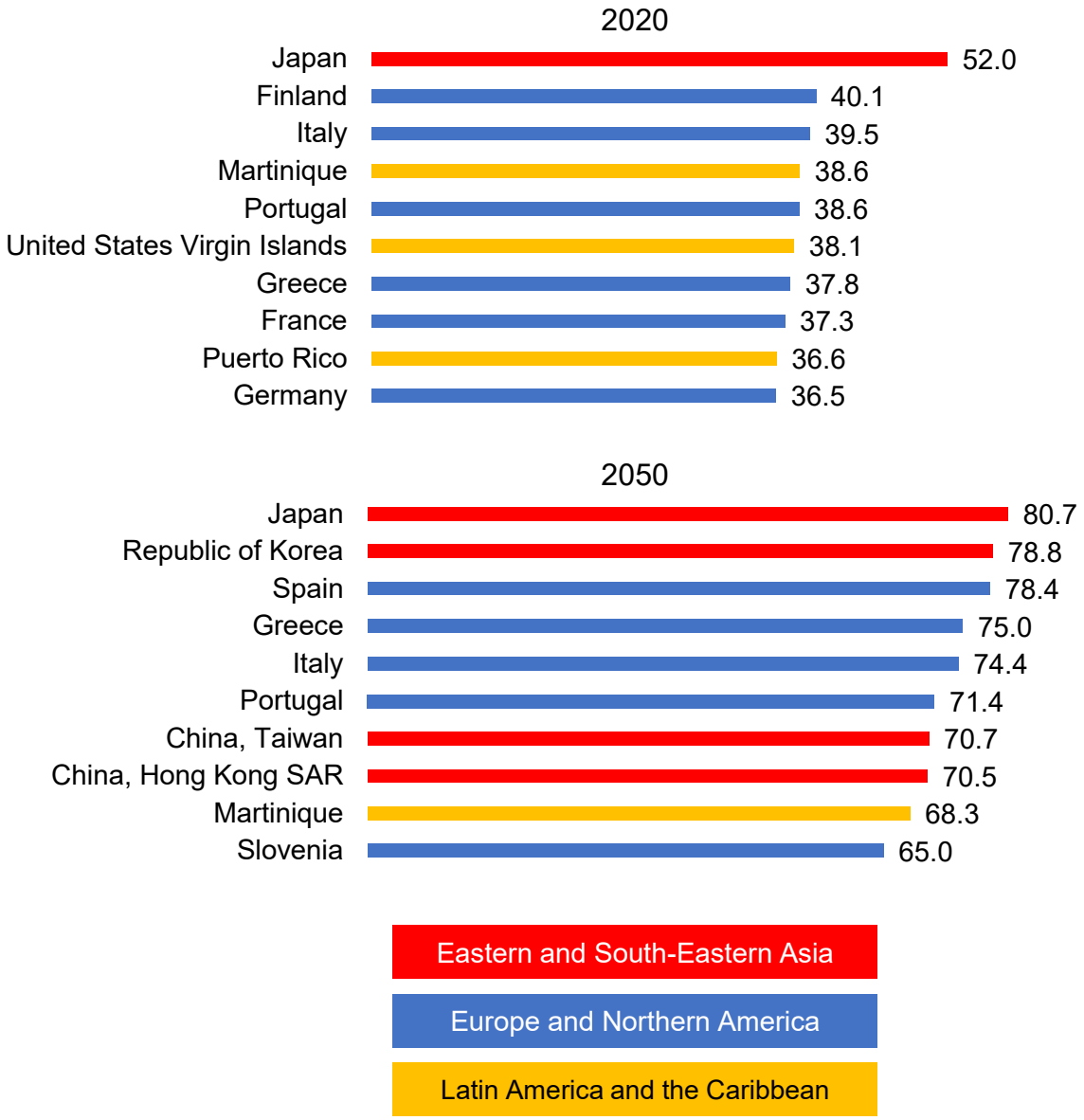

Figure 1-2 Top 10 countries or areas with the highest OADR in 2020 and by 2050.
Data source: United Nations, World Population Prospects 2019.

1.2 Importance of Blood Pressure Control

Aging consists of the progressive accumulation of changes in the body that increases susceptibility to disorders such as diabetes, dyslipidemia, dementia and cardiovascular disease (CVD), including hypertension. Hypertension is a highly prevalent condition with numerous health risks [2]. In 2015, 1.13 billion people worldwide were hypertensive [3,4].

According to World Health Organization, hypertension is defined as, systolic blood pressure (SBP) ≥ 140 mmHg and/or the diastolic blood pressure (DBP) ≥ 90 mmHg when measured on two different days. Similarly, in Japan, individuals with blood pressure (BP) levels of 140/90 mmHg or more are regarded as hypertensive. In 2019, the classification of BP levels was updated from the 2014 version to lower the target value of normal BP [5] (Table 1-1 and 1-2). In 2019, the subclasses “normal BP” and “high-normal BP” employed in 2014 were classified and expressed as “high-normal BP” and “elevated BP,” respectively.

Hypertension is one of the most important risk factors for CVD along with dyslipidemia and diabetes. CVD is the leading cause of death in the world. CVD accounts for approximately one-third of all deaths worldwide, with more than 17.9 million deaths per year in 2016 [6]. Also, ischemic heart disease and stroke, which are classified as CVD, are the leading and second leading causes of death (Figure 1-3).

Hypertension has also been recognized as an important risk factor for the development of cognitive decline and dementia [7–10]. Though cognitive function may be affected by hypertension through increased risk for stroke [11], hypertension is considered to have a causal relationship with dementia [12–14]. For example, the Framingham study, a long-term cardiovascular cohort study in the United States, showed that higher SBP and DBP in stroke-

free individuals in midlife were associated with worse performance on a composite global cognitive score and measures of attention and memory [15]. Similarly, in the Honolulu-Asia Aging Study, having a high SBP in midlife was associated with a two-fold increased risk of cognitive decline on a global cognitive test 25 years later [16]. In brief, these studies consistently showed that having high SBP or hypertension in midlife was associated with worse cognitive performance in late life. Thus, hypertension is highly associated with serious diseases such as CVD and dementia, so adequate control of BP is one of the most important issues to consider in an aging society.

Table 1-1 Classification of BP levels in Japanese Society of Hypertension Guidelines for the Managements of Hypertension 2014.

Classification	BP (mmHg)		
	SBP		DBP
Normal-range BP			
Optimal BP	< 120	and	< 80
Normal BP	120–129	and/or	80–84
High-normal BP	130–139	and/or	85–89
Hypertension			
Grade 1 hypertension	140–159	and/or	90–99
Grade 2 hypertension	160–179	and/or	100–109
Grade 3 hypertension	≥ 180	and/or	≥ 110
(Isolated) systolic hypertension	≥ 140	and	< 90

BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Table 1-2 Classification of BP levels in Japanese Society of Hypertension Guidelines for the Managements of Hypertension 2019.

Classification	BP (mmHg)		
	SBP		DBP
Normal-range BP			
Normal BP	< 120	and	< 80
High normal BP	120–129	and	< 80
Elevated BP	130–139	and/or	80–89
Hypertension			
Grade 1 hypertension	140–159	and/or	90–99
Grade 2 hypertension	160–179	and/or	100–109
Grade 3 hypertension	≥ 180	and/or	≥ 110
(Isolated) systolic hypertension	≥ 140	and	< 90

BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure.

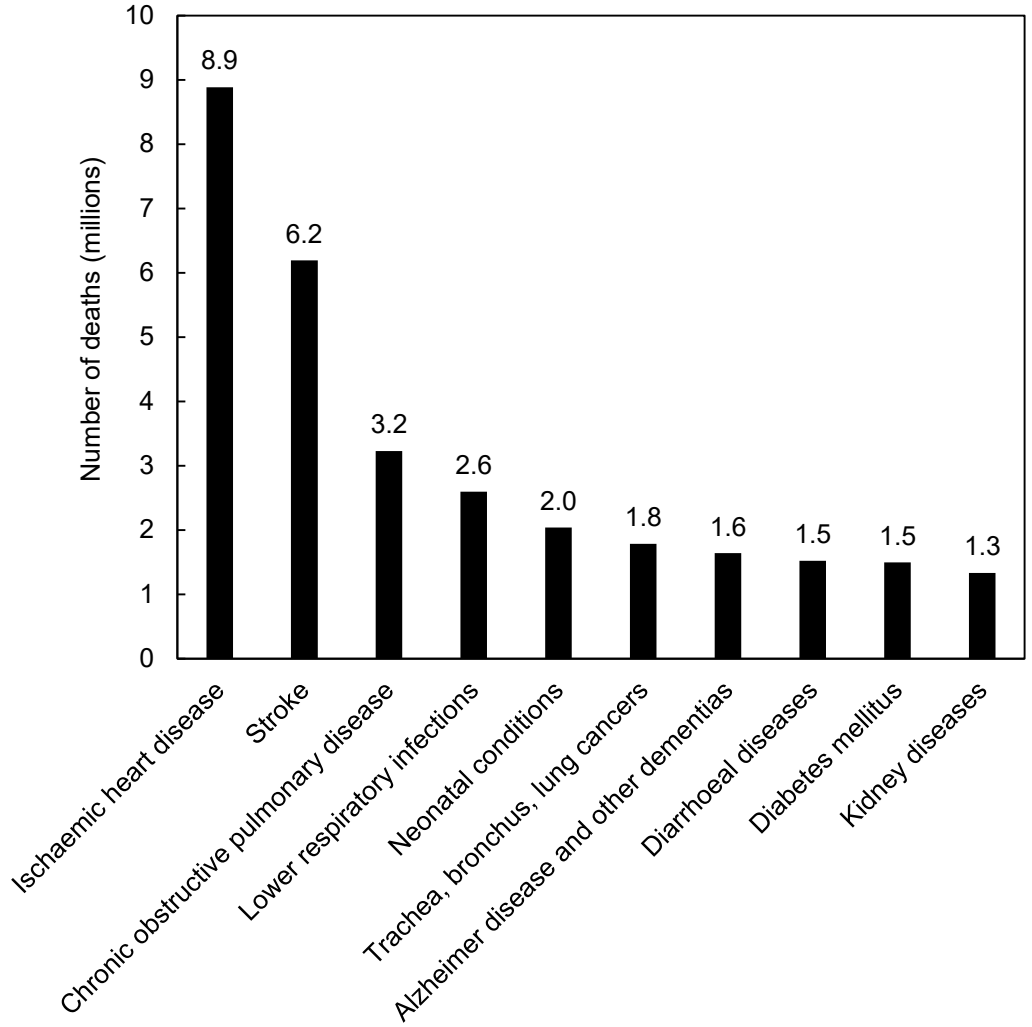

Figure 1-3 Global top 10 causes of death in 2019.

Data source: World Health Organization, Global Health Estimates 2020.

1.3 Angiotensin I-Converting Enzyme Inhibition

Angiotensin I-converting enzyme (ACE) inhibition is one of the effective ways to lower BP. ACE contributes to the formation of the vasoconstrictor angiotensin II (Ang II) and the degradation of the vasodilator bradykinin [17] (Figure 1-4), thereby making it key in the regulation of the renin-angiotensin system (RAS) and the kallikrein-kinin system.

The RAS plays critical roles in maintaining normal BP. Angiotensinogen, renin, angiotensin I (Ang I), ACE, Ang II, and Ang II receptors are involved in BP regulation by the RAS [18]. Recently, the family of the RAS has expanded to include peptides consisting of angiotensin 1–9 (Ang (1–9)), angiotensin 2–8 (Ang III), angiotensin 3–8 (Ang IV), angiotensin 1–7 (Ang (1–7)), and alamandine. Renin cleaves angiotensinogen, a protein with 452 amino acids, to produce Ang I [19]. Ang I is a decapeptide, which is then cleaved by ACE to produce the octapeptide Ang II [19]. Ang II causes vasoconstriction by stimulating Ang II type 1 receptor (AT1R) present on the vasculature [20]. Ang (1–9) is generated from Ang I by several carboxypeptidase-type enzymes and acts antagonistically to Ang II [21]. Ang III is produced from Ang II by enzymatic cleavage with aminopeptidase A and shows the same physiological response as Ang II [22]. Ang IV is cleaved from Ang III by aminopeptidase N and contributes to vasodilation, and learning and memory [23]. Ang (1–7) is formed by the catalytic action of ACE2 on Ang II and exerts its action through a G-protein-coupled receptor, Mas [24]. The effects of Ang (1–7)/Mas receptor axis activation provides equilibrium to the RAS system by promoting an antagonistic effect on the responses elicited by Ang II/AT1R [25]. Alamandine (Ala^1-Ang (1–7)) is synthesized from Ang (1–7) and has similar functional properties as Ang (1–7) [26].

Ang II is a key player in hypertension because vasoconstriction is caused by activation of AT1R. The effects of Ang II are observed in various organs including the kidneys, heart, and vascular system [27]. In the kidneys, AT1R regulates salt and water homeostasis, by stimulating the formation of aldosterone. In the heart, enhanced activation of AT1R promotes cardiac remodeling and aggravates the progression of heart failure. In the vascular system, AT1R stimulates vasoconstriction and the synthetic vascular smooth muscle phenotype, thereby contributes to the pathophysiology of hypertension. Based on these pathophysiologic processes, inhibition of Ang II generation by an ACE inhibitor has been important approach in the treatment of hypertension.

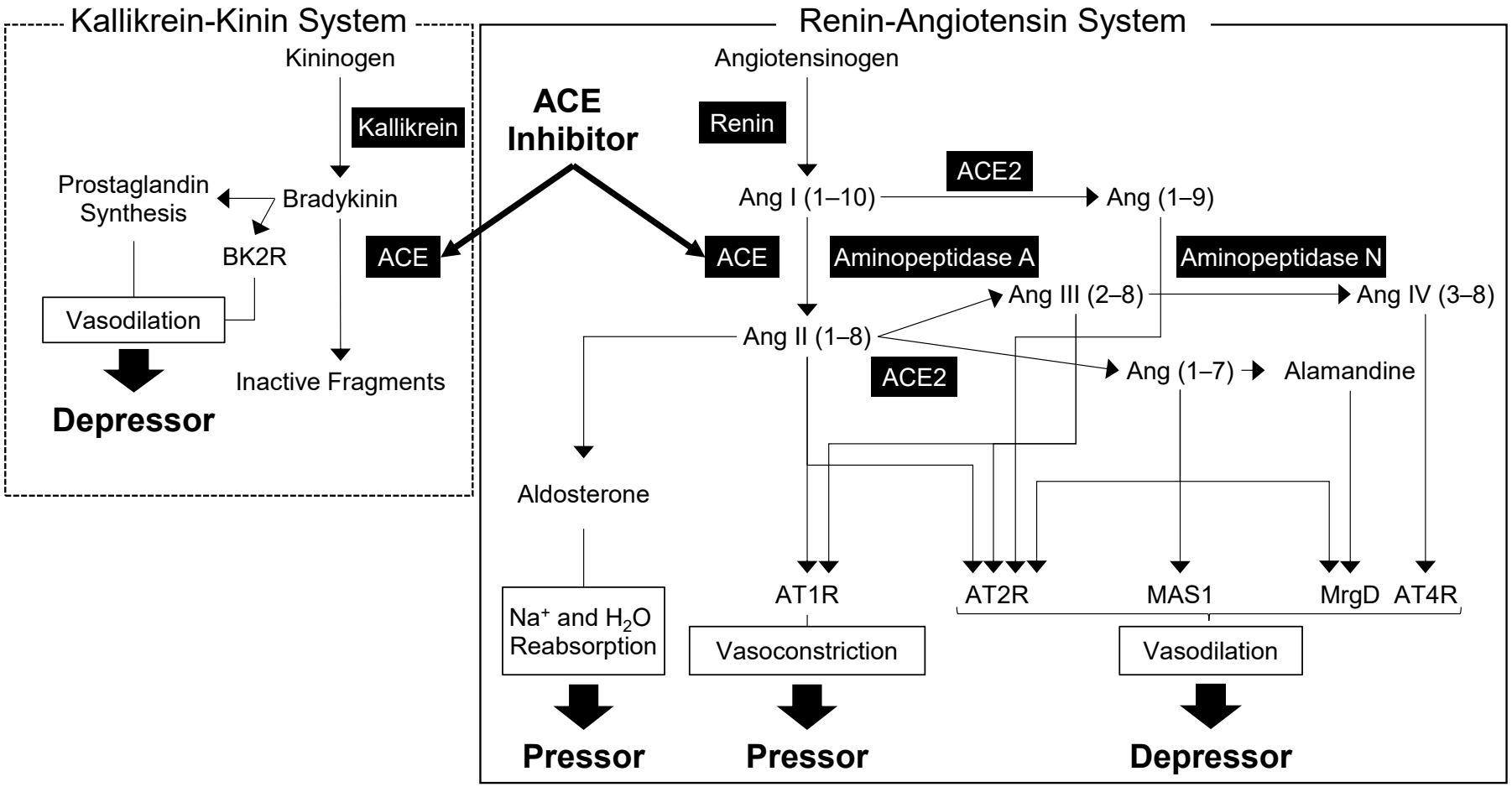

Figure 1-4 BP regulation by the renin-angiotensin and the kallikrein-kinin systems, and the inhibitory action of ACE inhibitors.

1.4 Dementia and Angiotensin I-Converting Enzyme Inhibition

Prevention and care for dementia such as Alzheimer's disease (AD) have become urgent worldwide issues. With the aging of the global society, the prevalence of dementia in adults is increasing and it is the seventh leading cause of death worldwide (Figure 1-3). The number of individuals living with dementia is predicted to increase from 50 million in 2015 to 152 million by 2050 [28]. In addition, the symptoms of dementia worsen over time making it a burden to patients, families, and caregivers.

AD is the most frequent form of dementia [29–31]. Three acetylcholinesterase inhibitors donepezil, galantamine and rivastigmine and *N*-methyl-D-aspartate receptor antagonist memantine are shown to improve cognitive function in patients with AD [32–34]. Although these drugs provide a moderate effect, they do not sufficiently alter the condition [35,36]. In addition, AD progresses for decades before symptoms appear; and when symptoms become clinically apparent, the condition is already too advanced for treatment [37,38]. Therefore, it is recommended that the intervention should precede onset of clinical symptoms [39–41].

The neuropathological hallmarks in the brains of AD patients include accumulation of senile plaques composed of aggregated amyloid-beta (Aβ), and neurofibrillary tangles composed of hyper-phosphorylated tau [42,43]. Formation of Aβ plaque occurs due to age-induced neurodegeneration, while neurofibrillary tangles of hyper-phosphorylated tau could form due to aberrant Aβ accumulation [44–46]. Based on this hypothesis, treatment approaches were developed, which interfere with Aβ production and/or enhance Aβ clearance and degradation. However, major clinical trials targeting Aβ showed no improvement in dementia,

so this concept is currently under discussion [47–51].

Although hypertension is a risk factor for dementia [52], centrally acting ACE inhibitors may reduce the risk of dementia or slow its progression, independent of their BP lowering effects [53–56]. The brain contains its own intrinsic RAS in addition to the peripheral one [57–60]. Activation of the sympathetic nervous system is considered to be one of the major functions of the brain RAS [61]. Several components of the neuronal RAS are altered in AD brains, such as an increase in the neuronal levels of the Ang II generated by ACE [62–64].

ACE in the central nervous system exerts its neurodegenerative activity through the generation of reactive oxygen species by AT1R stimulation of Ang II [65,66]. Furthermore, the activation of AT1R by Ang II promotes neuroinflammation. Intracerebroventricular (ICV) injection of Ang II impaired cognitive function in a mouse model of AD associated with hippocampal inflammation, oxidative stress, and increased Aβ deposition [67]. Considering these processes, the inhibition of Ang II generation by ACE inhibitors has been the promising approach in the treatment of cognitive decline and AD. Hence, centrally acting ACE inhibitors such as captopril, lisinopril, and perindopril could be considered for the prevention of neurodegeneration and dementia in patients at risk [68,69]. Several clinical studies have shown that the inhibition of ACE retards the process of neurodegeneration leading to cognitive decline and onset of AD [56,70–74]. The results of these clinical studies suggest that centrally active ACE inhibitors may have neuroprotective activity.

1.5 Angiotensin I-Converting Enzyme Inhibitory Effect of Milk-Derived Peptide

Bioactive peptides are specific protein fragments that have a positive effect on body functions or health conditions. Numerous peptides have been identified with various bioactivities. More than 4000 different bioactive peptides have been reported in a database BIOPEP-UWM [75]. These peptides encrypted in proteins are inactive. However, when released from their parental protein by enzymatic hydrolysis, microbial fermentation, or gastrointestinal digestion, they may regulate the human physiology by influencing several body systems [76].

Milk proteins are the most important sources of bioactive peptides [77–79]. They have been considered as the prominent candidates for various health-promoting functional foods targeting the cardiovascular, gastrointestinal, immune, and nervous systems [76,77] (Figure 1-5). For example, milk-derived peptides were reported to have anti-hypertensive, anti-thrombotic, anti-hypercholesterolemic, calcium-binding, anti-microbial, anti-appetizing, insulin regulation, anti-oxidant, opioid, cognitive improvement, immunomodulatory, and cytomodulatory effects [76,77,80,81].

ACE inhibition is one of the most studied functions of milk-derived peptides [82]. These peptides have attracted much attention because it has been suggested that they can be used as a safer alternative to control hypertension [76]. Numerous milk-derived peptides with ACE inhibitory activities have been identified (Table 1-3). For example, ACE inhibitory peptides have been isolated and characterized from αS1-casein [83–86], αS2-casein [87,88], β-casein [89–96], κ-casein [90,97], β-lactoglobulin [98–101], α-lactalbumin [92,101], and lactoferrin [102] (Table 1-3). Among them, β-casein-derived lactotripeptides Val-Pro-Pro (VPP) and Ile-

Pro-Pro (IPP) have been extensively studied. They were identified from sour milk fermented with *Lactobacillus helveticus* and *Saccharomyces cervisiae* [93]. The half maximal inhibitory concentration (IC_{50}) values of VPP and IPP were 5 and 9 μM, respectively. Subsequently, several studies regarding to the BP lowering effect of the lactotripeptides have been carried out, mainly in spontaneously hypertensive rats (SHRs) [103,104] and in humans [105–111]. Turpeinen et al. analyzed 19 clinical trials with 1532 prehypertensive or mildly hypertensive individuals and concluded that the overall anti-hypertensive effect of lactotripeptides was −4.0 mmHg in SBP and −1.9 mmHg in DBP [107]. When restricted to Asian populations only, the overall BP lowering effect of lactotripeptides consumption was even higher: −5.6 mmHg in SBP and −2.6 mmHg in DBP. However, one meta-analysis with only Europeans revealed a slightly lower BP lowering effect: −1.28 mmHg in SBP and −0.59 mmHg in DBP [109]. On the other hand, considering the potential bias and the statistically significant publication biases, the anti-hypertensive effect of the lactotripeptides is still under discussion, and further studies are warranted [110].

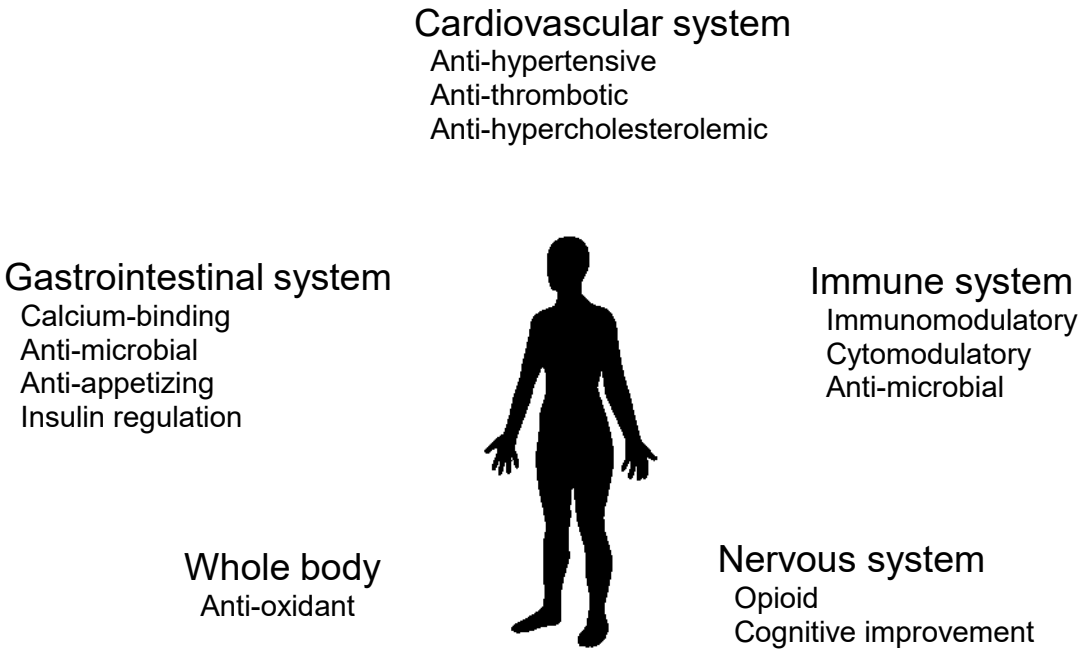

Figure 1-5 Physiological functions of milk-derived peptides.

Table 1-3 Milk-derived peptides with ACE inhibitory activities.

Peptide	Protein ID	Protein	Position	IC_{50} (μM)	Reference
RPKHPIKHQ	P02662	αS1-casein	16–24	13.4	[83]
FFVAP	P02662	αS1-casein	38–42	6.0	[84]
RYLGY	P02662	αS1-casein	105–109	0.7	[85]
AYFYPEL	P02662	αS1-casein	158–164	6.6	[85]
YY	P02662	αS1-casein	180–181	9.0	[86]
FALPQY	P02663	αS2-casein	189–194	4.3	[87]
TVY	P02663	αS2-casein	197–199	15.0	[87]
MKP	P02663	αS2-casein	205–207	0.4	[88]
KVLILA	P02666	β-casein	2–7	20.9	[89]
RINKK	P02666	β-casein	40–44	18.3	[90]
LVYPFP	P02666	β-casein	73–78	132.0	[91]
VYPFPGPIPNSLPQNIPP	P02666	β-casein	74–91	87.0	[92]
SLPQN	P02666	β-casein	84–88	9.5	[90]
IPP	P02666	β-casein	89–91	5.0	[93]
VPP	P02666	β-casein	99–101	9.0	[93]
LQP	P02666	β-casein	103–105	3.4	[94]
MAP	P02666	β-casein	117–119	0.8	[94]
SQSKVLPVPQ	P02666	β-casein	181–190	92.0	[95]
VLGPVRGPFP	P02666	β-casein	212–221	137.0	[96]
IAK	P02668	κ-casein	43–45	15.7	[97]
ARHPH	P02668	κ-casein	117–121	15.2	[90]
LDIQK	P02754	β-lactoglobulin	26–30	27.6	[98]
WY	P02754	β-lactoglobulin	35–36	38.3	[99]
LKALPMH	P02754	β-lactoglobulin	156–162	11.0	[100]
ALPMHIR	P02754	β-lactoglobulin	158–164	42.6	[101]
GYGGVSLPEW	P00711	α-lactalbumin	36–45	2.0	[100]
YGGVSLPEW	P00711	α-lactalbumin	37–45	16.0	[92]
FKCRRWQW	P24627	lactoferrin	36–43	10.5	[102]
FKCRRWQWR	P24627	lactoferrin	36–44	2.9	[102]
CRRWQWR	P24627	lactoferrin	38–44	2.3	[102]

1.6 Tripeptide Met-Lys-Pro

Met-Lys-Pro (MKP), the bovine αS2-casein-derived tripeptide, was identified from the casein hydrolysate as the peptide with the highest contribution to the ACE inhibitory effect [112]. In fact, MKP showed stronger inhibitory activity (IC_{50} = 0.43 μM) compared to other food-derived ACE inhibitory peptides *in vitro* [88]. Oral administration of casein hydrolysate containing MKP (casein-derived MKP) inhibited the elevated BP of SHRs [88,112], as well as ingestion of synthetic MKP [88]. Additionally, the orally administered ^{14}C-MKP can be absorbed and transferred intact into the bloodstream in SHRs [88]. Furthermore, *ex vivo*, the pre-treatment of MKP inhibited Ang II-dependent vasoconstriction of thoracic aorta of rats [88]. These results indicated that orally administered MKP might lower BP through intestinal absorption and ACE inhibition. Therefore, MKP was considered to be a promising candidate for casein-derived anti-hypertensive peptide.

Casein-derived MKP significantly attenuated cognitive decline in an *in vivo* study [113]. AD model mice induced by ICV injection of Aβ42 were orally administered casein-derived MKP, and changes in cognitive function were evaluated using the Morris water maze. In addition, the hippocampus was collected after behavioral testing, and the inflammatory cytokine and nicotinamide adenine dinucleotide phosphate oxidase subunit expression was measured. The results showed that daily administration of casein-derived MKP significantly attenuated Aβ42-induced cognitive decline and reduced Aβ42-induced tumor necrosis factor-α, monocyte chemoattractant protein-1, inducible nitric oxide synthase, $p47^{phox}$, and $gp91^{phox}$ expression. Improvement in cognitive function was also observed with the administration of synthetic MKP alone. These results indicated that MKP could act as a therapeutic agent of

cognitive function. Therefore, MKP may be a casein-derived peptide that improves both BP and cognitive function.

1.7 Objectives of This Study

The utilization of foods with valuable bioactivity is beneficial for disease prevention and healthy life expectancy. On the other hand, it is essential to demonstrate the efficacy and safety of these foods in humans. Based on the results of *in vitro* and *in vivo* studies, casein-derived MKP has BP lowering and cognitive improvement effects. However, there was not much information on their efficacy and safety in humans. Hence, the aim of the present study was to evaluate the effects of casein-derived MKP on human BP, cognitive function, and safety. In particular, the present study focused on the preventive effects of casein-derived MKP, and included healthy participants and those with mild symptoms. First, to investigate the effectiveness of casein-derived MKP for reducing BP, a 12-week randomized controlled trial (RCT) was conducted in participants with high-normal BP or grade 1 hypertension. Second, to assess the ability of casein-derived MKP for improving cognitive function, a 24-week RCT was conducted in a community-dwelling adults without dementia. Finally, to evaluate the safety of high-dose intake of casein-derived MKP, a 4-week RCT was conducted in healthy adults.

References

[1] World Health Organization. World Population Prospects 2019. https://population.un.org/wpp/. Accessed February 17, 2021.

[2] GBD 2019 Risk Factors Collaborators. Global burden of 87 risk factors in 204 countries and territories, 1990–2019: a systematic analysis for the Global Burden of Disease Study 2019. *Lancet*. 2020;396(10258):1223–1249. doi:10.1016/S0140-6736(20)30752-2

[3] Mrowka R. Recent advances in hypertension research. *Acta Physiol (Oxf)*. 2019;226(4):e13295. doi:10.1111/apha.13295

[4] World Health Organization. Global Health Observatory. https://www.who.int/data/gho. Accessed February 17, 2021.

[5] Umemura S, Arima H, Arima S, et al. The Japanese Society of Hypertension Guidelines for the Management of Hypertension (JSH 2019). *Hypertens Res*. 2019;42(9):1235–1481. doi:10.1038/s41440-019-0284-9

[6] World Health Organization. Cardiovascular diseases (CVDs). https://www.who.int/en/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds). Accessed February 17, 2021.

[7] Cherubini A, Lowenthal DT, Paran E, Mecocci P, Williams LS, Senin U. Hypertension and cognitive function in the elderly. *Dis Mon*. 2010;56(3):106–147. doi:10.1016/j.disamonth.2009.12.007

[8] DeCarli C. Blood pressure control and cognitive performance: something to think about with aging. *JAMA*. 2015;313(19):1963–1964. doi:10.1001/jama.2015.3113

[9] Hanon O, Forette F. Treatment of hypertension and prevention of dementia. *Alzheimers Dement*. 2005;1(1):30–37. doi:10.1016/j.jalz.2005.06.022

[10] Power MC, Weuve J, Gagne JJ, McQueen MB, Viswanathan A, Blacker D. The association between blood pressure and incident Alzheimer disease: a systematic review and meta-analysis. *Epidemiology*. 2011;22(5):646–659. doi:10.1097/EDE.0b013e31822708b5

[11] Ihle-Hansen H, Thommessen B, Fagerland MW, et al. Blood pressure control to prevent decline in cognition after stroke. *Vasc Health Risk Manag*. 2015;11:311–316. doi:10.2147/VHRM.S82839

[12] Frishman WH. Are antihypertensive agents protective against dementia? A review of clinical and preclinical data. *Heart Dis*. 2002;4(6):380–386. doi:10.1097/00132580-200211000-00007

[13] Manolio TA, Olson J, Longstreth WT. Hypertension and cognitive function: pathophysiologic effects of hypertension on the brain. *Curr Hypertens Rep*. 2003;5(3):255–261. doi:10.1007/s11906-003-0029-6

[14] Buford TW. Hypertension and aging. *Ageing Res Rev*. 2016;26:96–111. doi:10.1016/j.arr.2016.01.007

[15] Elias MF, Wolf PA, D'Agostino RB, Cobb J, White LR. Untreated blood pressure level is inversely related to cognitive functioning: the Framingham Study. *Am J Epidemiol*. 1993;138(6):353–364. doi:10.1093/oxfordjournals.aje.a116868

[16] Launer LJ, Masaki K, Petrovitch H, Foley D, Havlik RJ. The association between midlife blood pressure levels and late-life cognitive function. The Honolulu-Asia Aging Study. *JAMA*. 1995;274(23):1846–1851. doi:10.1001/jama.1995.03530230032026

[17] Li GH, Le GW, Shi YH, Shrestha S. Angiotensin I–converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects. *Nutrition research*. 2004;24(7):469–486. doi:10.1016/j.nutres.2003.10.014

[18] Lu H, Cassis LA, Kooi CW, Daugherty A. Structure and functions of angiotensinogen. *Hypertens Res*. 2016;39(7):492–500. doi:10.1038/hr.2016.17

[19] Karnik SS, Unal H, Kemp JR, et al. International Union of Basic and Clinical Pharmacology. XCIX. Angiotensin Receptors: Interpreters of Pathophysiological Angiotensinergic Stimuli. *Pharmacol Rev*. 2015;67(4):754–819. doi:10.1124/pr.114.010454

[20] Lavoie JL, Sigmund CD. Minireview: overview of the renin-angiotensin system—an endocrine and paracrine system. *Endocrinology*. 2003;144(6):2179–2183. doi:10.1210/en.2003-0150

[21] Ocaranza MP, Michea L, Chiong M, Lagos CF, Lavandero S, Jalil JE. Recent insights and therapeutic perspectives of angiotensin-(1–9) in the cardiovascular system. *Clin Sci (Lond)*. 2014;127(9):549–557. doi:10.1042/CS20130449

[22] Yugandhar VG, Clark MA. Angiotensin III: a physiological relevant peptide of the renin angiotensin system. *Peptides*. 2013;46:26–32. doi:10.1016/j.peptides.2013.04.014

[23] Haulica I, Bild W, Serban DN. Angiotensin peptides and their pleiotropic actions. *J Renin Angiotensin Aldosterone Syst*. 2005;6(3):121–131. doi:10.3317/jraas.2005.018

[24] Bader M. ACE2, angiotensin-(1–7), and Mas: the other side of the coin. *Pflugers Arch*. 2013;465(1):79–85. doi:10.1007/s00424-012-1120-0

[25] Ferrario CM. New physiological concepts of the renin-angiotensin system from the investigation of precursors and products of angiotensin I metabolism. *Hypertension*. 2010;55(2):445–452. doi:10.1161/HYPERTENSIONAHA.109.145839

[26] Carey RM. Newly discovered components and actions of the renin-angiotensin system. *Hypertension*. 2013;62(5):818–822. doi:10.1161/HYPERTENSIONAHA.113.01111

[27] Forrester SJ, Booz GW, Sigmund CD, et al. Angiotensin II Signal Transduction: An Update on Mechanisms of Physiology and Pathophysiology. *Physiol Rev*. 2018;98(3):1627–1738. doi:10.1152/physrev.00038.2017

[28] World Health Organization. Risk reduction of cognitive decline and dementia. https://www.who.int/mental_health/neurology/dementia/guidelines_risk_reduction/en/. Published 2019. Accessed February 21, 2021.

[29] Gorelick PB, Scuteri A, Black SE, et al. Vascular contributions to cognitive impairment and dementia: a statement for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke*. 2011;42(9):2672–2713. doi:10.1161/STR.0b013e3182299496

[30] Matsui Y, Tanizaki Y, Arima H, et al. Incidence and survival of dementia in a general population of Japanese elderly: the Hisayama study. *J Neurol Neurosurg Psychiatry*. 2009;80(4):366–370. doi:10.1136/jnnp.2008.155481

[31] Abbott A. Dementia: a problem for our age. *Nature*. 2011;475(7355):S2–S4. doi:10.1038/475S2a

[32] Tan CC, Yu JT, Wang HF, et al. Efficacy and safety of donepezil, galantamine, rivastigmine, and memantine for the treatment of Alzheimer's disease: a systematic review and meta-analysis. *J Alzheimers Dis*. 2014;41(2):615–631. doi:10.3233/JAD-132690

[33] Li DD, Zhang YH, Zhang W, Zhao P. Meta-analysis of randomized controlled trials on the efficacy and safety of donepezil, galantamine, rivastigmine, and memantine for the treatment of Alzheimer's disease. *Front Neurosci*. 2019;13:472. doi:10.3389/fnins.2019.00472

[34] Kulshreshtha A, Piplani P. Current pharmacotherapy and putative disease-modifying therapy for Alzheimer's disease. *Neurol Sci*. 2016;37(9):1403–1435. doi:10.1007/s10072-016-2625-7

[35] Anand R, Gill KD, Mahdi AA. Therapeutics of Alzheimer's disease: Past, present and future. *Neuropharmacology*. 2014;76 Pt A:27–50. doi:10.1016/j.neuropharm.2013.07.004

[36] Schneider LS, Mangialasche F, Andreasen N, et al. Clinical trials and late-stage drug development for Alzheimer's disease: an appraisal from 1984 to 2014. *J Intern Med*. 2014;275(3):251–283. doi:10.1111/joim.12191

[37] Jack CR Jr, Knopman DS, Jagust WJ, et al. Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. *Lancet Neurol*. 2010;9(1):119–128. doi:10.1016/S1474-4422(09)70299-6

[38] Sperling RA, Aisen PS, Beckett LA, et al. Toward defining the preclinical stages of Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement*. 2011;7(3):280–292. doi:10.1016/j.jalz.2011.03.003

[39] McDade E, Bateman RJ. Stop Alzheimer's before it starts. *Nature*. 2017;547(7662):153–155. doi:10.1038/547153a

[40] Crous-Bou M, Minguillón C, Gramunt N, Molinuevo JL. Alzheimer's disease prevention: from risk factors to early intervention. *Alzheimers Res Ther*. 2017;9(1):71. doi:10.1186/s13195-017-0297-z

[41] Livingston G, Sommerlad A, Orgeta V, et al. Dementia prevention, intervention, and care. *Lancet*. 2017;390(10113):2673–2734. doi:10.1016/S0140-6736(17)31363-6

[42] Masters CL, Beyreuther K. Alzheimer's centennial legacy: prospects for rational therapeutic intervention targeting the Abeta amyloid pathway. *Brain*. 2006;129(Pt 11):2823–2839. doi:10.1093/brain/awl251

[43] Ballard C, Gauthier S, Corbett A, Brayne C, Aarsland D, Jones E. Alzheimer's disease. *Lancet*. 2011;377(9770):1019–1031. doi:10.1016/S0140-6736(10)61349-9

[44] Lesné SE, Sherman MA, Grant M, et al. Brain amyloid-β oligomers in ageing and Alzheimer's disease. *Brain*. 2013;136(Pt 5):1383–1398. doi:10.1093/brain/awt062

[45] Gómez-Isla T, Hollister R, West H, et al. Neuronal loss correlates with but exceeds neurofibrillary tangles in Alzheimer's disease. *Ann Neurol*. 1997;41(1):17–24. doi:10.1002/ana.410410106

[46] Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science*. 2002;297(5580):353–356. doi:10.1126/science.1072994

[47] Doody RS, Thomas RG, Farlow M, et al. Phase 3 trials of solanezumab for mild-to-moderate Alzheimer's disease. *N Engl J Med*. 2014;370(4):311–321. doi:10.1056/NEJMoa1312889

[48] Honig LS, Vellas B, Woodward M, et al. Trial of solanezumab for mild dementia due to Alzheimer's disease. *N Engl J Med*. 2018;378(4):321–330. doi:10.1056/NEJMoa1705971

[49] Salloway S, Sperling R, Fox NC, et al. Two phase 3 trials of bapineuzumab in mild-to-moderate Alzheimer's disease. *N Engl J Med*. 2014;370(4):322–333. doi:10.1056/NEJMoa1304839

[50] Vandenberghe R, Rinne JO, Boada M, et al. Bapineuzumab for mild to moderate Alzheimer's disease in two global, randomized, phase 3 trials. *Alzheimers Res Ther*. 2016;8(1):18. doi:10.1186/s13195-016-0189-7

[51] Egan MF, Kost J, Tariot PN, et al. Randomized trial of verubecestat for mild-to-moderate Alzheimer's disease. *N Engl J Med*. 2018;378(18):1691–1703. doi:10.1056/NEJMoa1706441

[52] Kivipelto M, Helkala EL, Laakso MP, et al. Midlife vascular risk factors and Alzheimer's disease in later life: longitudinal, population based study. *BMJ*. 2001;322(7300):1447–1451. doi:10.1136/bmj.322.7300.1447

[53] Shah K, Qureshi SU, Johnson M, Parikh N, Schulz PE, Kunik ME. Does use of antihypertensive drugs affect the incidence or progression of dementia? A systematic review. *Am J Geriatr Pharmacother*. 2009;7(5):250–261. doi:10.1016/j.amjopharm.2009.11.001

[54] Davies NM, Kehoe PG, Ben-Shlomo Y, Martin RM. Associations of anti-hypertensive treatments with Alzheimer's disease, vascular dementia, and other dementias. *J Alzheimers Dis*. 2011;26(4):699–708. doi:10.3233/JAD-2011-110347

[55] Qiu WQ, Mwamburi M, Besser LM, et al. Angiotensin converting enzyme inhibitors and the reduced risk of Alzheimer's disease in the absence of apolipoprotein E4 allele. *J Alzheimers Dis*. 2013;37(2):421–428. doi:10.3233/JAD-130716

[56] O'Caoimh R, Healy L, Gao Y, et al. Effects of centrally acting angiotensin converting enzyme inhibitors on functional decline in patients with Alzheimer's disease. *J Alzheimers Dis*. 2014;40(3):595–603. doi:10.3233/JAD-131694

[57] McKinley MJ, Albiston AL, Allen AM, et al. The brain renin-angiotensin system: location and physiological roles. *Int J Biochem Cell Biol*. 2003;35(6):901–918. doi:10.1016/s1357-2725(02)00306-0

[58] Wright JW, Harding JW. The brain renin-angiotensin system: a diversity of functions and implications for CNS diseases. *Pflugers Arch*. 2013;465(1):133–151. doi:10.1007/s00424-012-1102-2

[59] Grobe JL, Xu D, Sigmund CD. An intracellular renin-angiotensin system in neurons: fact, hypothesis, or fantasy. *Physiology (Bethesda)*. 2008;23:187–193. doi:10.1152/physiol.00002.2008

[60] Saavedra JM. Brain angiotensin II: new developments, unanswered questions and therapeutic opportunities. *Cell Mol Neurobiol*. 2005;25(3–4):485–512. doi:10.1007/s10571-005-4011-5

[61] Campese VM, Shaohua Y, Huiquin Z. Oxidative stress mediates angiotensin II-dependent stimulation of sympathetic nerve activity. *Hypertension*. 2005;46(3):533–539. doi:10.1161/01.HYP.0000179088.57586.26

[62] Arregui A, Perry EK, Rossor M, Tomlinson BE. Angiotensin converting enzyme in Alzheimer's disease increased activity in caudate nucleus and cortical areas. *J Neurochem*. 1982;38(5):1490–1492. doi:10.1111/j.1471-4159.1982.tb07930.x

[63] Savaskan E, Hock C, Olivieri G, et al. Cortical alterations of angiotensin converting enzyme, angiotensin II and AT1 receptor in Alzheimer's dementia. *Neurobiol Aging*. 2001;22(4):541–546. doi:10.1016/s0197-4580(00)00259-1

[64] Miners JS, Ashby E, Van Helmond Z, et al. Angiotensin-converting enzyme (ACE) levels and activity in Alzheimer's disease, and relationship of perivascular ACE-1 to cerebral amyloid angiopathy. *Neuropathol Appl Neurobiol*. 2008;34(2):181–193. doi:10.1111/j.1365-2990.2007.00885.x

[65] Dong YF, Kataoka K, Tokutomi Y, et al. Perindopril, a centrally active angiotensin-converting enzyme inhibitor, prevents cognitive impairment in mouse models of Alzheimer's disease. *FASEB J*. 2011;25(9):2911–2920. doi:10.1096/fj.11-182873

[66] Zimmerman MC, Lazartigues E, Lang JA, et al. Superoxide mediates the actions of angiotensin II in the central nervous system. *Circ Res*. 2002;91(11):1038–1045. doi:10.1161/01.res.0000043501.47934.fa

[67] Takane K, Hasegawa Y, Lin B, et al. Detrimental effects of centrally administered angiotensin II are enhanced in a mouse model of Alzheimer disease independently of blood pressure. *J Am Heart Assoc*. 2017;6(4):e004897. doi:10.1161/JAHA.116.004897

[68] Kehoe PG. The coming of age of the angiotensin hypothesis in Alzheimer's disease: Progress toward disease prevention and treatment? *J Alzheimers Dis*. 2018;62(3):1443–1466. doi:10.3233/JAD-171119

[69] Fazal K, Perera G, Khondoker M, Howard R, Stewart R. Associations of centrally acting ACE inhibitors with cognitive decline and survival in Alzheimer's disease. *BJPsych Open*. 2017;3(4):158–164. doi:10.1192/bjpo.bp.116.004184

[70] Sink KM, Leng X, Williamson J, et al. Angiotensin-converting enzyme inhibitors and cognitive decline in older adults with hypertension: results from the Cardiovascular Health Study. *Arch Intern Med*. 2009;169(13):1195–1202. doi:10.1001/archinternmed.2009.175

[71] Zhuang S, Wang HF, Wang X, Li J, Xing CM. The association of renin-angiotensin system blockade use with the risks of cognitive impairment of aging and Alzheimer's disease: A meta-analysis. *J Clin Neurosci*. 2016;33:32–38. doi:10.1016/j.jocn.2016.02.036

[72] Soto ME, van Kan GA, Nourhashemi F, et al. Angiotensin-converting enzyme inhibitors and Alzheimer's disease progression in older adults: results from the Réseau sur la Maladie d'Alzheimer Français cohort. *J Am Geriatr Soc*. 2013;61(9):1482–1488. doi:10.1111/jgs.12415

[73] Gao Y, O'Caoimh R, Healy L, et al. Effects of centrally acting ACE inhibitors on the rate of cognitive decline in dementia. *BMJ Open*. 2013;3(7):e002881. doi:10.1136/bmjopen-2013-002881

[74] Ohrui T, Tomita N, Sato-Nakagawa T, et al. Effects of brain-penetrating ACE inhibitors on Alzheimer disease progression. *Neurology*. 2004;63(7):1324–1325. doi:10.1212/01.wnl.0000140705.23869.e9

[75] Minkiewicz P, Iwaniak A, Darewicz M. BIOPEP-UWM database of bioactive peptides: current opportunities. *Int J Mol Sci*. 2019;20(23):5978. doi:10.3390/ijms20235978

[76] Mohanty DP, Mohapatra S, Misra S, Sahu PS. Milk derived bioactive peptides and their impact on human health - A review. *Saudi J Biol Sci*. 2016;23(5):577–583. doi:10.1016/j.sjbs.2015.06.005

[77] Korhonen H. Milk-derived bioactive peptides: From science to applications. *J Funct Foods*. 2009;1(2):177–187. doi:10.1016/j.jff.2009.01.007

[78] Sánchez A, Vázquez A. Bioactive peptides: A review. *Food Qual Saf*. 2017;1(1):29–46. doi:10.1093/fqs/fyx006

[79] Meisel H, FitzGerald RJ. Biofunctional peptides from milk proteins: mineral binding and cytomodulatory effects. *Curr Pharm Des*. 2003;9(16):1289–1295. doi:10.2174/1381612033454847

[80] Phelan M, Aherne A, FitzGerald RJ, O'Brien NM. Casein-derived bioactive peptides: Biological effects, industrial uses, safety aspects and regulatory status. *Int Dairy J*. 2009;19(11):643–654. doi:10.1016/j.idairyj.2009.06.001

[81] Ricci-Cabello I, Herrera MO, Artacho R. Possible role of milk-derived bioactive peptides in the treatment and prevention of metabolic syndrome. *Nutr Rev*. 2012;70(4):241–255. doi:10.1111/j.1753-4887.2011.00448.x

[82] Jäkälä P, Vapaatalo H. Antihypertensive Peptides from Milk Proteins. *Pharmaceuticals (Basel)*. 2010;3(1):251–272. doi:10.3390/ph3010251

[83] Saito T, Nakamura T, Kitazawa H, Kawai Y, Itoh T. Isolation and structural analysis of antihypertensive peptides that exist naturally in Gouda cheese. *J Dairy Sci*. 2000;83(7):1434–1440. doi:10.3168/jds.S0022-0302(00)75013-2

[84] Maruyama S, Nakagomi K, Tomizuka N, Suzuki H. Angiotensin I-converting enzyme inhibitor derived from an enzymatic hydrolysate of casein. II. Isolation and bradykinin-potentiating activity on the uterus and the ileum of rats. *Agric and Biol Chem*. 1985;49(5):1405–1409. doi:10.1080/00021369.1985.10866901

[85] del Mar Contreras M, Carrón R, Montero MJ, Ramos M, Recio I. Novel casein-derived peptides with antihypertensive activity. *Int Dairy J*. 2009;19(10):566–573. doi:10.1016/j.idairyj.2009.05.004

[86] Fuglsang A, Nilsson D, Nyborg NC. Characterization of new milk-derived inhibitors of angiotensin converting enzyme *in vitro* and *in vivo*. *J Enzyme Inhib Med Chem*. 2003;18(5):407–412. doi:10.1080/1475636031000138723

[87] Tauzin J, Miclo L, Gaillard JL. Angiotensin-I-converting enzyme inhibitory peptides from tryptic hydrolysate of bovine alphaS2-casein. *FEBS Lett*. 2002;531(2):369–374. doi:10.1016/s0014-5793(02)03576-7

[88] Yamada A, Sakurai T, Ochi D, Mitsuyama E, Yamauchi K, Abe F. Antihypertensive effect of the bovine casein-derived peptide Met-Lys-Pro. *Food Chem*. 2015;172:441–446. doi:10.1016/j.foodchem.2014.09.098

[89] Wang ZL, Zhang SS, Wei WANG, Feng FQ, Shan WG. A novel angiotensin I converting enzyme inhibitory peptide from the milk casein: Virtual screening and docking studies. *Agric Sci China*. 2011;10(3):463–467. doi:10.1016/S1671-2927(11)60026-6

[90] Donkor ON, Henriksson A, Singh TK, Vasiljevic T, Shah NP. ACE-inhibitory activity of probiotic yoghurt. *Int Dairy J*. 2007;17(11):1321–1331. doi:10.1016/j.idairyj.2007.02.009

[91] Gonzalez-Gonzalez C, Gibson T, Jauregi P. Novel probiotic-fermented milk with angiotensin I-converting enzyme inhibitory peptides produced by *Bifidobacterium bifidum* MF 20/5. *Int J Food Microbiol.* 2013;167(2):131–137. doi:10.1016/j.ijfoodmicro.2013.09.002

[92] Otte J, Shalaby SM, Zakora M, Nielsen MS. Fractionation and identification of ACE-inhibitory peptides from α-lactalbumin and β-casein produced by thermolysin-catalysed hydrolysis. *Int Dairy J*. 2007;17(12):1460–1472. doi:10.1016/j.idairyj.2007.04.008

[93] Nakamura Y, Yamamoto N, Sakai K, Okubo A, Yamazaki S, Takano T. Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. *J Dairy Sci*. 1995;78(4):777–783. doi:10.3168/jds.S0022-0302(95)76689-9

[94] Tonouchi H, Suzuki M, Uchida M, Oda M. Antihypertensive effect of an angiotensin converting enzyme inhibitory peptide from enzyme modified cheese. *J Dairy Res*. 2008;75(3):284–290. doi:10.1017/S0022029908003452

[95] Hayes M, Stanton C, Slattery H, et al. Casein fermentate of *Lactobacillus animalis* DPC6134 contains a range of novel propeptide angiotensin-converting enzyme inhibitors. *Appl Environ Microbiol*. 2007;73(14):4658–4667. doi:10.1128/AEM.00096-07

[96] Quirós A, Ramos M, Muguerza B, et al. Identification of novel antihypertensive peptides in milk fermented with *Enterococcus faecalis*. *Int Dairy J*. 2007;17(1):33–41. doi:10.1016/j.idairyj.2005.12.011

[97] Gómez-Ruiz JA, Ramos M, Recio I. Identification of novel angiotensin-converting enzyme-inhibitory peptides from ovine milk proteins by CE-MS and chromatographic techniques. *Electrophoresis*. 2007;28(22):4202–4211. doi:10.1002/elps.200700324

[98] Hernández-Ledesma B, Ramos M, Recio I, Amigo L. Effect of beta-lactoglobulin hydrolysis with thermolysin under denaturing temperatures on the release of bioactive peptides. *J Chromatogr A*. 2006;1116(1-2):31–37. doi:10.1016/j.chroma.2006.03.006

[99] Hernández-Ledesma B, Amigo L, Recio I, Bartolomé B. ACE-inhibitory and radical-scavenging activity of peptides derived from beta-lactoglobulin f(19–25). Interactions with ascorbic acid. *J Agric Food Chem*. 2007;55(9):3392–3397. doi:10.1021/jf063427j

[100] Lacroix IM, Meng G, Cheung IW, Li-Chan EC. Do whey protein-derived peptides have dual dipeptidyl-peptidase IV and angiotensin I-converting enzyme inhibitory activities? *J Funct Foods*. 2016;21:87–96. doi:10.1016/j.jff.2015.11.038

[101] Mullally MM, Meisel H, FitzGerald RJ. Identification of a novel angiotensin-I-converting enzyme inhibitory peptide corresponding to a tryptic fragment of bovine beta-lactoglobulin. *FEBS Lett*. 1997;402(2–3):99–101. doi:10.1016/s0014-5793(96)01503-7

[102] Ruiz-Giménez P, Ibáñez A, Salom JB, et al. Antihypertensive properties of lactoferricin B-derived peptides. *J Agric Food Chem*. 2010;58(11):6721–6727. doi:10.1021/jf100899u

[103] Nakamura Y, Yamamoto N, Sakai K, Takano T. Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors to angiotensin I-converting enzyme. *J Dairy Sci*. 1995;78(6):1253–1257. doi:10.3168/jds.S0022-0302(95)76745-5

[104] Masuda O, Nakamura Y, Takano T. Antihypertensive peptides are present in aorta after oral administration of sour milk containing these peptides to spontaneously hypertensive rats. *J Nutr*. 1996;126(12):3063–3068. doi:10.1093/jn/126.12.3063

[105] Xu JY, Qin LQ, Wang PY, Li W, Chang C. Effect of milk tripeptides on blood pressure: a meta-analysis of randomized controlled trials. *Nutrition*. 2008;24(10):933–940. doi:10.1016/j.nut.2008.04.004

[106] Cicero AF, Gerocarni B, Laghi L, Borghi C. Blood pressure lowering effect of lactotripeptides assumed as functional foods: a meta-analysis of current available clinical trials. *J Hum Hypertens*. 2011;25(7):425–436. doi:10.1038/jhh.2010.85

[107] Turpeinen AM, Järvenpää S, Kautiainen H, Korpela R, Vapaatalo H. Antihypertensive effects of bioactive tripeptides-a random effects meta-analysis. *Ann Med*. 2013;45(1):51–56. doi:10.3109/07853890.2012.663926

[108] Qin LQ, Xu JY, Dong JY, Zhao Y, van Bladeren P, Zhang W. Lactotripeptides intake and blood pressure management: a meta-analysis of randomised controlled clinical trials. *Nutr Metab Cardiovasc Dis*. 2013;23(5):395–402. doi:10.1016/j.numecd.2013.02.006

[109] Cicero AF, Aubin F, Azais-Braesco V, Borghi C. Do the lactotripeptides isoleucine-proline-proline and valine-proline-proline reduce systolic blood pressure in European subjects? A meta-analysis of randomized controlled trials. *Am J Hypertens*. 2013;26(3):442–449. doi:10.1093/ajh/hps044

[110] Fekete ÁA, Givens DI, Lovegrove JA. Casein-derived lactotripeptides reduce systolic and diastolic blood pressure in a meta-analysis of randomised clinical trials. *Nutrients*. 2015;7(1):659–681. doi:10.3390/nu7010659

[111] Chanson-Rolle A, Aubin F, Braesco V, Hamasaki T, Kitakaze M. Influence of the lactotripeptides isoleucine-proline-proline and valine-proline-proline on systolic blood pressure in Japanese subjects: A systematic review and meta-analysis of randomized controlled trials. *PLoS One*. 2015;10(11):e0142235. doi:10.1371/journal.pone.0142235

[112] Yamada A, Sakurai T, Ochi D, Mitsuyama E, Yamauchi K, Abe F. Novel angiotensin I-converting enzyme inhibitory peptide derived from bovine casein. *Food Chem*. 2013;141(4):3781–3789. doi:10.1016/j.foodchem.2013.06.089

[113] Min LJ, Kobayashi Y, Mogi M, et al. Administration of bovine casein-derived peptide prevents cognitive decline in Alzheimer disease model mice. *PLoS One*. 2017;12(2):e0171515. doi:10.1371/journal.pone.0171515

Chapter 2

Effect of Casein-Derived Met-Lys-Pro on Blood Pressure in Participants With High-Normal Blood Pressure or Grade 1 Hypertension: A Randomized Controlled Trial

2.1 Introduction

High BP is the most important risk factor for CVD such as myocardial infraction, stroke, and heart failure [1]. One in four adults worldwide in 2000 was hypertensive, and one in three adults worldwide is expected to have this condition by 2025 [2]. Thus, prevention and management of hypertension have become public health priorities.

Dietary improvement is recommended for people with mild hypertension [3]. For instance, the intake of low-fat milk and dairy products has been shown to reduce BP [4]. The Dietary Approaches to Stop Hypertension trial suggested that a diet rich in fruits, vegetables, and low-fat dairy foods and with reduced saturated and total fat can substantially lower BP [5]. The large-scale Rotterdam study also concluded that intake of low-fat dairy products can contribute to the prevention of hypertension in the elderly [6].

Milk-derived peptides have widely been pursued for controlling elevated BP [7]. Many of these peptides have demonstrated *in vitro* inhibitory effect on ACE, the central regulator of the RAS controlling fluid volume. Noted milk-derived peptides with ACE inhibitory effect include the lactotripeptides VPP and IPP found in fermented or enzymatically hydrolyzed milk [8–11]. The bovine milk protein hydrolysate C12 peptide has also been shown to reduce BP in clinical trials [12,13]. However, relatively fewer identified milk-derived peptides have been tested for anti-hypertensive effects in humans [14,15].

MKP was identified as a tripeptide derived from bovine casein hydrolysate [16] with strong ACE inhibitory activity i*n vitro* [17]. Orally administered casein-derived MKP reduced BP in SHRs [17], providing expectation on the BP lowering effect in humans. In fact, a small number of preliminary clinical trial suggested that casein-derived MKP may lower BP in

participants with elevated BP [18].

The primary objective of this chapter was to investigate the effectiveness of casein-derived MKP for reducing BP in participants with high-normal BP or grade 1 hypertension. The secondary objective was to obtain preliminary information on the effect of this ingredient on the brachial-ankle pulse wave velocity (baPWV), a useful predictor of CVD risk [1,19,20].

2.2 Methods

2.2.1 Participants

Adult volunteers living in Tokyo, and surrounding areas were recruited through the website and emails. Recruitment was conducted between October and November 2016. Those wishing to participate contacted the study coordinator, who explained the study project to them. Inclusion criteria included: age of 30–65 years, high-normal BP (SBP from 130 to 139 mmHg and/or DBP from 85 to 89 mmHg) or grade 1 hypertension (SBP from 140 to 159 mmHg and/or DBP from 90 to 99 mmHg) [1] (Table 1-1), and body mass index (BMI, in kg/m^2) above 18.5 and less than 30.0. Exclusion criteria included: receiving treatment for serious diseases (e.g., heart failure, myocardial infarction, malignant tumor) ; a history of serious disease; currently under medication for diabetes, hypertension, or dyslipidemia; a history of serious allergies to medicine or food; pregnancy, lactation, or planning to get pregnant during the study period; heavy smoking, alcohol abuse, or unstable lifestyle; ineligible due to physician's diagnosis based on participant background, physical examination, and interview. To determine eligibility, participants were asked to complete health and lifestyle questionnaires.

The study protocol was examined and approved by the institutional review board and the

Ethics Committee of the Japan Conference of Clinical Research (approved on October 20, 2016). Study protocols were conducted in accordance with the Declaration of Helsinki. Before beginning the study, all participants provided written informed consent and were informed that they were free to withdraw at any time without obligation. This trial was registered at the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) as UMIN000024786.

2.2.2 Study Products

All study products were delivered in gelatin-based capsules. The composition of the study products is detailed in Table 2-1. The MKP-containing capsules were made from casein hydrolysate manufactured by the Morinaga Milk Industry (Tokyo, Japan) [16]. Each capsule contained 50 μg MKP in 0.25 g casein hydrolysate. The placebo capsules contained 0.25 g sodium caseinate with no detectable MKP content. MKP and placebo capsules were matched for appearance.

Table 2-1 Composition of the study products.

Components	MKP (2 capsules/day)	Placebo (2 capsules/day)
Energy (kcal)	2.5	2.5
Protein (g)	0.6	0.6
Fat (g)	0.0	0.0
Carbohydrates (g)	0.0	0.0
Sodium (mg)	4.0	6.0
Casein hydrolysate containing MKP (g/day)	0.5	0.0
MKP (μg/day)	100.0	0.0
Sodium caseinate (g/day)	0.0	0.5

2.2.3 Procedures

The study was designed as a randomized, placebo-controlled, double-blind parallel-group trial. The primary outcome of the study was change in seated office SBP and DBP from baseline to week 12. Participants were randomly assigned to receive MKP or placebo capsules using computer-generated lists of random numbers via the randomly permuted block method. The participants in the MKP and placebo groups were instructed to consume two capsules daily at their leisure, respectively. The participants, the physician on record, the researchers assessing outcomes, and the researchers performing statistical analyses were blinded to the treatment group allocation.

The trial was conducted in Tokyo, Japan, between October 2016 and March 2017. The study period comprised 4 weeks for pre-treatment observation, 12 weeks for treatment, and 2 weeks for post-treatment observation. Six study visits were scheduled: at weeks −4 and −1 (the pre-treatment observation period); weeks 4, 8, and 12 (the treatment period); and week +2 (the

post-treatment observation period). The BP value at week −1 was taken as the baseline value. Eligibility was assessed on the basis of self-reported data from health and lifestyle questionnaires, physician interviews, and physical examination at week −1. Eligible participants were randomly assigned to either the MKP or placebo group at week −1. Enrolled participants started the study protocol within one week after eligibility was confirmed. During the study period, participants were asked to maintain their typical daily activities and diet. Participants were also asked to keep a record using a diary, which included items related to supplementation of study products, stress conditions, change of the quantity of meal, amount of alcohol consumed, physical activities, medications, and hospital visits. Before visiting for BP measurements, participants were asked to do the following: not take alcohol the day before the test day, not smoking or bathing on measurement day until after measurements, completing defecation more than one hour before measurement, visiting without intake of study products, and eating meals two hours or more before measurement. Study staff interviewed participants before and throughout the study to ensure compliance with these lifestyle requirements based on participant diaries. Treatment compliance was assessed by counting the number of capsules returned at the time of the final study visit and checking participant diaries. The following conditions were defined as noncompliance: a significant change in lifestyle (continued to increase/decrease in diet volume, alcohol consumption, and/or excessive change in the amount of daily exercise), continual excessive stress, less than 80% ingestion rate, mismatch between the number of study product intake declared in the diary and that which remained, and ingestion of medication affecting BP.

2.2.4 Physical Examination

Body height was measured once during the pre-treatment period (week −4) and was used to calculate BMI during the study period. Body weight (BW) was measured six times; twice during the pre-treatment observation period (weeks −4 and −1), three times during the treatment period (weeks 4, 8, and 12), and once during the post-treatment observation period (week +2), to calculate BMI.

2.2.5 Blood Pressure Measurement

BP was determined at all visits using a calibrated and validated digital sphygmomanometer with appropriately sized cuffs (Omron HEM-907; Omron Healthcare Co., Ltd., Kyoto, Japan). BP was measured with the participant in a seated position and their arm supported at heart level following a > 5 min rest period. Multiple measurements were recorded at 2 min intervals and the mean of the last two was used in the analysis. For baseline (week −1) assessment, BP was measured on both arms, and the arm with the higher mean reading was used in subsequent visits.

2.2.6 Pulse Wave Measurement

The baPWV was measured in participants aged 50 years or older at weeks −1, 4, 8, and 12 using a BP-203RPE III automatic pulse wave analyzer (Omron Healthcare Co., Ltd., Kyoto, Japan) in accordance with the manufacturer's instructions. Participants were examined in the supine position with four cuffs wrapped around both the brachia and ankles. After each pulse wave to the brachia and ankles was measured, the baPWV value was calculated according to the delay

from the start of the brachial pulse wave to the start of the ankle pulse wave. The distances from the heart to the brachium and ankle were calculated based on participant body height.

2.2.7 Safety Monitoring

All participants were monitored throughout the study for adverse events (AEs) and side-effects. Safety monitoring consisted of a questionnaire that inquired about general health and occurrence of any health-related events. The relation of AEs to intake of study products was determined by the physician on record while remaining blinded to group allocation. The severity of AEs was evaluated according to the Common Terminology Criteria for Adverse Events (CTCAE) version 3.0 JCOG/JSCO.

2.2.8 Sample Size

The sample size required to detect a mean SBP difference of 6 mmHg with standard deviation (SD) of 10 mmHg (Cohen's $d = 0.6$) at $\alpha = 0.05$ and power = 0.8 by unpaired *t*-test was calculated as 45 study participants per group, totaling 90 participants in the study. Considering a 25% drop out rate, 60 participants per group would need to be recruited.

2.2.9 Statistical Analysis

Data were presented as mean ± standard error (SE) except for baseline (pre-treatment) values, which were expressed as mean ± SD. The purpose of this study was not to elucidate the influence of lifestyle but the accurate assessment of efficacy of study products. Therefore, analyses were conducted for per protocol set, with the exclusion of data on participants that

were non-compliant. Differences between baseline and post-baseline values were analyzed by paired *t*-test. Unpaired *t*-test was used to compare results between the groups. BP data were also analyzed according to severity by dividing participants into high-normal BP and grade 1 hypertension subgroups. All statistical analyses were performed using SAS 9.4 Software (SAS Institute Japan Ltd., Tokyo, Japan), were two-tailed, and statistical significance was established at $P < 0.05$.

2.3 Results

2.3.1 Participants

Of the 753 candidates recruited, 120 were enrolled and randomly allocated to the MKP or placebo group (Figure 2-1). Two participants withdrew before further assessment for personal reasons unrelated to the trial. Fifteen participants (seven in the MKP group and eight in the placebo group) discontinued intervention for the following reasons: nine participants (five in the MKP group and four in the placebo group) reported significant lifestyle changes such as exercise habits, food style, and drinking amount during the trial; four participants (one in the MKP group and three in the placebo group) had intake rate that did not match the remaining number of capsules; one participant in the MKP group reported taking a medication affecting BP; one participant in the placebo group reported frequent stress. The overall dropout rate was 14.2% (17 of 120). Table 2-2 shows the baseline characteristics of 103 participants (53 in the MKP group and 50 in the placebo group) who were retained for analysis.

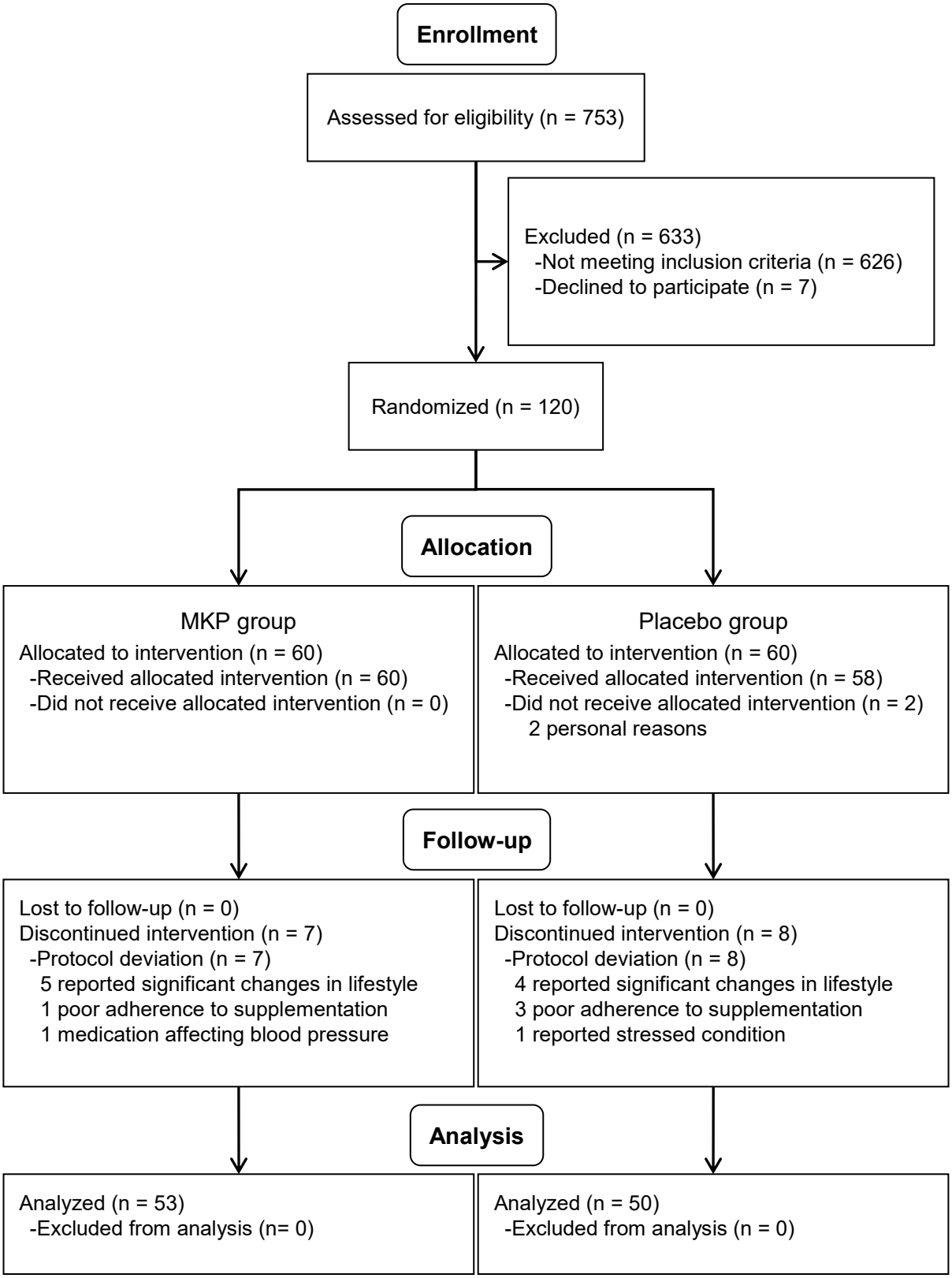

Figure 2-1 Study flow diagram.

Table 2-2 Baseline characteristics of the participants.

Characteristic	MKP (n = 53)	Placebo (n = 50)
Male/Female (n)	39/14	39/11
Age (years)	50.8 ± 7.8	50.5 ± 7.6
BMI (kg/m^2)	24.6 ± 2.7	23.4 ± 2.5
High-normal BP/Grade 1 hypertension	31/22	30/20
SBP (mmHg)	137.5 ± 7.3	136.5 ± 7.3
DBP (mmHg)	86.0 ± 7.5	86.5 ± 6.9
Pulse rate (beats/min)	74.3 ± 11.3	74.9 ± 10.0

Data represent numbers of participants or mean ± SD. BMI, body mass index; BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure.

2.3.2 Blood Pressure

The mean SBP values at baseline, during treatment and post-treatment for the MKP and placebo groups are shown in Table 2-3. Mean SBP in the MKP group differed significantly from baseline at both week 12 (P = 0.0002) and post-treatment (P = 0.0015), while mean DBP in the MKP group did not differ from baseline. Mean SBP in the placebo group differed significantly from baseline at post-treatment (P = 0.0116), while mean DBP in the placebo group did not differ from baseline. Although there were no significant differences between the MKP and placebo groups at any time point, both mean SBP and DBP showed the largest effect sizes at week 12 (Cohen's d = 0.26 and 0.29, respectively). The change in SBP from baseline to week 12 (Figure 2-2) was significantly greater in the MKP group compared to the placebo group (mean change with 95% CI: −3.9 [−7.1, −0.7] mmHg, P = 0.0173).

The results of subgroup analysis are shown in Tables 2-4, 2-5, and Figure 2-3. In

participants with high-normal BP, mean SBP in the MKP group differed significantly from baseline at week 12 (P = 0.0064) and post-treatment (P = 0.0309). Mean DBP in the placebo group was significantly higher at week 12 than at baseline (P = 0.0271). Mean DBP was significantly lower in the MKP group than in the placebo group at week 12 (mean difference with 95% CI: −4.4 [−8.5, −0.3] mmHg, P = 0.0353). There were no other significant differences between the groups, however, the largest effect sizes for both mean SBP and DBP were observed at week 12 (Cohen's d = 0.38 and 0.54, respectively). The change in SBP from baseline to week 12 was significantly greater in the MKP group than in the placebo group (mean change with 95% CI: −4.4 [−8.6, −0.2] mmHg, P = 0.0414). For participants with grade 1 hypertension, mean SBP in the MKP group differed significantly from baseline at week 12 (P = 0.0103) and post-treatment (P = 0.0219). Mean SBP in the placebo group was significantly lower at post-treatment than at baseline (P = 0.0100). There were no other significant differences within the group and between the groups, respectively.

Table 2-3 BP of the participants.

Variable	Week	MKP			Placebo			*P* value between the groups	Cohen's *d*
		n	Mean ± SE	*P* value within the group	n	Mean ± SE	*P* value within the group		
SBP	Baseline	53	137.5 ± 1.0		50	136.5 ± 1.0		0.4790	
(mmHg)	4	53	135.8 ± 1.7	0.2450	50	136.6 ± 1.4	0.9160	0.6960	0.08
	8	51	136.4 ± 1.6	0.5480	48	133.9 ± 1.5	0.1230	0.2440	0.23
	12	53	132.5 ± 1.6	0.0002	49	135.1 ± 1.2	0.2850	0.1940	0.26
	Post	53	132.8 ± 1.5	0.0015	48	132.9 ± 1.3	0.0116	0.9460	0.01
DBP	Baseline	53	86.0 ± 1.0		50	86.5 ± 1.0		0.7420	
(mmHg)	4	53	87.8 ± 1.6	0.1080	50	87.8 ± 1.1	0.0846	0.9670	0.01
	8	51	86.2 ± 1.5	0.8410	48	87.1 ± 1.2	0.6210	0.6350	0.10
	12	53	85.0 ± 1.5	0.4010	49	87.7 ± 1.0	0.0658	0.1480	0.29
	Post	53	85.3 ± 1.4	0.4970	48	86.1 ± 1.1	0.9570	0.6840	0.08

Baseline values were measured at week −1. Post-treatment values were measured at week +2. BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure.

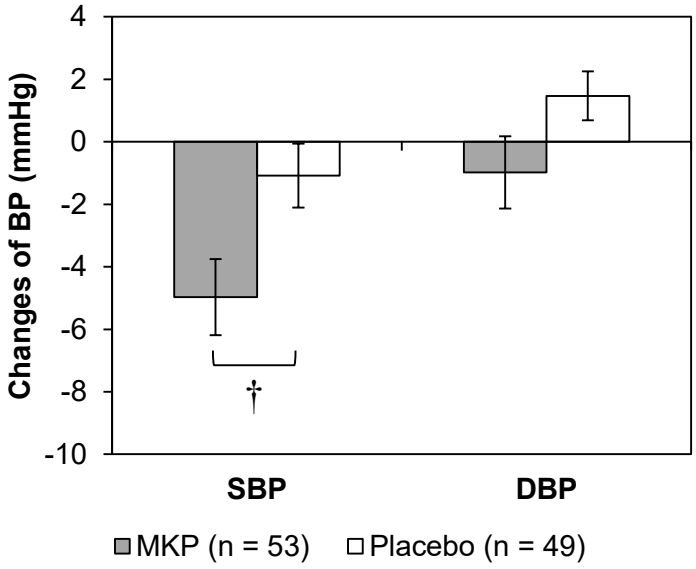

Figure 2-2 Changes of BP from baseline values at week 12.
Values are presented as mean ± SE. Baseline values were measured at week −1 (pre-treatment period). †$P < 0.05$: Significantly different from the placebo group (unpaired *t*-test). BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Table 2-4 BP of the participants with high-normal BP.

Variable	Week	MKP			Placebo			*P* value between the groups	Cohen's *d*
		n	Mean ± SE	*P* value within the group	n	Mean ± SE	*P* value within the group		
SBP	Baseline	31	133.5 ± 0.7		30	132.3 ± 0.8		0.2550	
(mmHg)	4	31	130.5 ± 1.8	0.1220	30	133.6 ± 1.8	0.4700	0.2340	0.31
	8	30	133.8 ± 1.9	0.8500	29	131.0 ± 1.9	0.5440	0.2950	0.28
	12	31	128.5 ± 1.8	0.0064	30	131.7 ± 1.2	0.6730	0.1420	0.38
	Post	31	129.8 ± 1.8	0.0309	30	130.7 ± 1.5	0.3160	0.7150	0.09
DBP	Baseline	31	81.6 ± 1.0		30	82.9 ± 1.0		0.3380	
(mmHg)	4	31	83.1 ± 1.6	0.3400	30	84.7 ± 1.1	0.0484	0.4090	0.21
	8	30	82.8 ± 1.6	0.3980	29	85.1 ± 1.4	0.1960	0.2900	0.28
	12	31	80.9 ± 1.7	0.6520	30	85.3 ± 1.2	0.0271	0.0353	0.54
	Post	31	81.7 ± 1.4	0.9240	30	84.1 ± 1.2	0.3530	0.2120	0.32

Baseline values were measured at week −1. Post-treatment values were measured at week +2. BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Table 2-5 BP of the participants with grade 1 hypertension.

Variable	Week	MKP			Placebo			*P* value between the groups	Cohen's *d*
		n	Mean ± SE	*P* value within the group	n	Mean ± SE	*P* value within the group		
SBP	Baseline	22	143.2 ± 1.5		20	142.9 ± 1.4		0.8650	
(mmHg)	4	22	143.2 ± 2.6	0.9920	20	141.2 ± 1.7	0.4230	0.5280	0.20
	8	21	140.2 ± 2.6	0.2890	19	138.3 ± 2.1	0.0902	0.5910	0.17
	12	22	138.2 ± 2.4	0.0103	19	140.4 ± 1.9	0.2910	0.4680	0.23
	Post	22	136.9 ± 2.5	0.0219	18	136.6 ± 2.2	0.0100	0.9260	0.03
DBP	Baseline	22	92.2 ± 1.2		20	91.8 ± 1.2		0.7950	
(mmHg)	4	22	94.5 ± 2.5	0.1750	20	92.3 ± 1.6	0.6890	0.4660	0.23
	8	21	91.0 ± 2.4	0.4430	19	90.1 ± 1.8	0.2710	0.7670	0.10
	12	22	90.8 ± 2.3	0.4460	19	91.6 ± 1.5	0.9480	0.7950	0.08
	Post	22	90.4 ± 2.4	0.3010	18	89.4 ± 1.9	0.2480	0.7350	0.11

Baseline values were measured at week −1. Post-treatment values were measured at week +2. BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure.

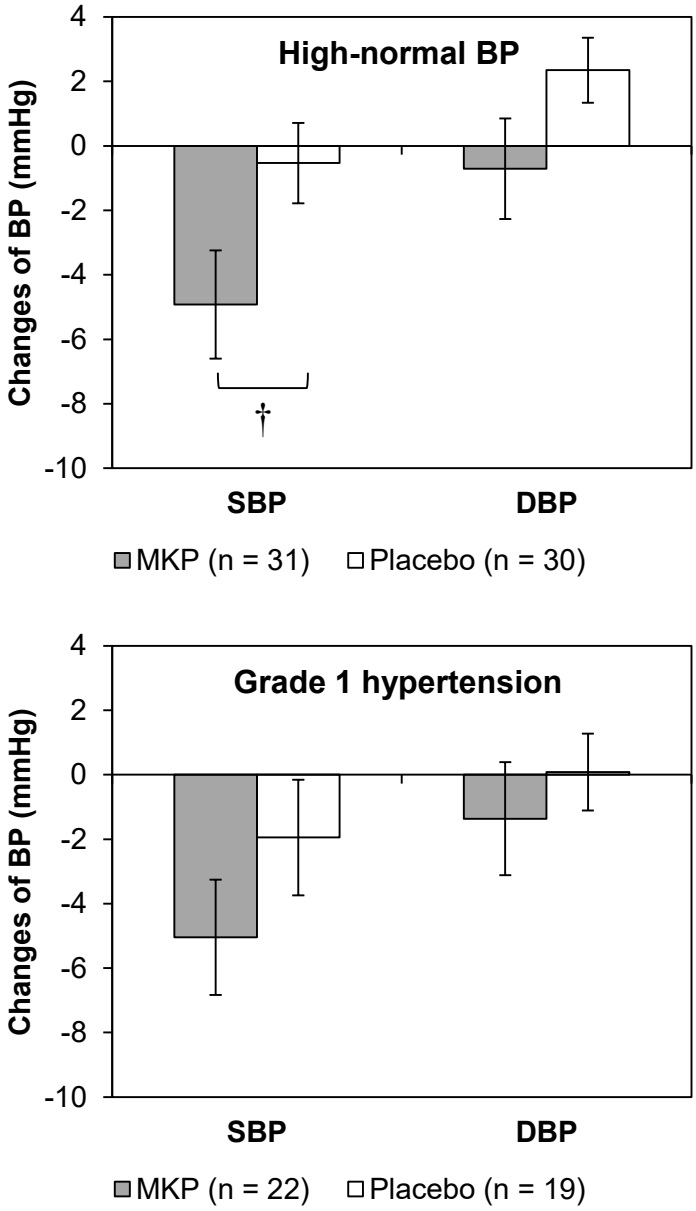

Figure 2-3 Subgroup analysis of changes of BP from baseline values at week 12.
Based on baseline values of BP, participants were divided into high-normal BP or grade 1 hypertension groups for analysis. Values are mean ± SE. Baseline values were measured at week −1 (pre-treatment period). †$P < 0.05$: Significantly different from the placebo group (unpaired *t*-test). BP, blood pressure; SBP, systolic blood pressure, DBP, diastolic blood pressure.

2.3.3 Brachial-Ankle Pulse Wave Velocity

Table 2-6 presents the baPWV values before, during, and after daily intake of MKP or placebo in participants aged 50 years or older. There was no significant effect of MKP treatment on baPWV.

Table 2-6 Brachial-ankle pulse wave velocity of the participants aged 50 years and above at baseline, during treatment, and post-treatment.

Variable	Week	MKP		Placebo	
		n	Mean ± SE	n	Mean ± SE
baPWV	Baseline	31	14.7 ± 0.3	26	15.2 ± 0.3
(right, m/s)	4	31	14.8 ± 0.3	27	15.2 ± 0.3
	8	29	14.6 ± 0.3	25	15.3 ± 0.4
	12	31	14.4 ± 0.3	26	15.2 ± 0.3
baPWV	Baseline	31	14.7 ± 0.3	26	15.1 ± 0.3
(left, m/s)	4	31	14.7 ± 0.3	27	15.1 ± 0.3
	8	29	14.5 ± 0.4	25	15.3 ± 0.4
	12	31	14.4 ± 0.3	26	15.1 ± 0.3

Baseline values were measured at week −1. No significant differences from both the baseline value and the placebo group were observed. baPWV; brachial-ankle pulse wave velocity.

2.3.4 Safety

Throughout the study, 61 AEs were reported by 37 participants, with 28 from 19 participants in the MKP group and 33 from 18 participants in the placebo group. AEs were as follows: 35 cold symptoms from 25 participants, 10 incidences of headache from seven participants, seven

gastrointestinal symptoms from four participants, four influenza symptoms from four participants, three seasonal allergies from three participants, one sprain, and one pain (calf). All the reported AEs were CTCAE grade 1 (mild) and unrelated to intake of study products.

2.4 Discussion

Daily ingestion of casein-derived MKP significantly lowered SBP in participants with high-normal BP or grade 1 hypertension while demonstrating good tolerability, with no treatment-related AEs reported over 12 weeks of treatment and 2 weeks post-treatment. Casein-derived MKP shows good water solubility and can be blended into foods rather than taken in capsule form. Therefore, regular dietary supplementation with casein-derived MKP may be a promising intervention for safely lowering BP and associated disease risks.

A meta-analysis of 16 cohort studies from Japan concluded that SBP was a more useful risk indicator of stroke and myocardial infarction than DBP, pulse pressure, or mean BP [21]. From a meta-analysis of 61 studies, a 2-mmHg reduction in SBP corresponded to about 10% decrease in stroke mortality and about 7% decrease in coronary heart disease mortality in middle age [22]. In the MKP group, SBP decreased by 3.9 mmHg compared to the placebo group after 12 weeks of ingestion, within the range considered sufficient to lower the risk of CVD.

It has been reported that the ACE inhibitory activity of MKP *in vitro* was comparable to other milk-derived peptides such as lactotripeptides [17]. Lactotripeptides are among the most extensively studied milk-derived peptides with BP-lowering effects. A meta-analysis of 18 clinical trials on lactotripeptides revealed a 3.73 mmHg (95% CI: −6.70, −1.76) drop in SBP

and 1.97 mmHg (95% CI: −3.85, −0.64) drop in DBP [11]. A direct comparison among lactotripeptides and casein-derived MKP is still required to assess the possible advantages of each in lowering BP among various hypertensive patient groups.

To accurately understand the potential benefits of bioactive peptides, it is important to evaluate their bioavailability [23,24]. Although ACE inhibition is one of the effective ways for lowering BP [25,26], in order to evaluate the anti-hypertensive effect of ACE inhibitory peptides, it is necessary to evaluate not only their activity but also their bioavailability *in vivo*. In fact, however, there are only a few reports on the bioavailability of ACE inhibitory peptides in animals and humans [24]. It was reported that anti-hypertensive dipeptide Val-Tyl (VY) with ACE inhibitory activity was detected in the plasma of SHRs [27] and humans [28,29] after oral ingestion. It was also known that orally administered VY was distributed not only in plasma but also in heart, liver, and kidney in SHRs [30]. In the case of MKP, it has been shown in SHRs that orally ingested ^{14}C-MKP was absorbed and transferred intact into the bloodstream [17]. Additionally, after oral ingestion of ^{14}C-MKP by SHRs, the results suggested that MKP or its metabolites may be distributed in many organs and tissues such as the small intestine, pancreas, and brain [31]. Considering that Ang II-dependent vasoconstriction of thoracic aorta of SHRs was inhibited by MKP pre-treatment [17], these results suggest that also in humans, orally ingested MKP may be transferred intact into the bloodstream and lowers SBP primarily via blockade of the RAS.

In contrast to SBP, regular casein-derived MKP ingestion had no effect on baPWV in participants over 50 years of age. This result may stem from the limited sample size relative to the detection sensitivity of baPWV or from the relatively brief (12 weeks) study period. In

addition to BP, age, and sex, baPWV could be affected by other risk factors such as blood glucose level and renal function not controlled in this study [32–34]. Furthermore, mean baPWV was well below 1.8 m/s, the cut-off predictor of CVD [35]. It is necessary to conduct larger-scale and longer clinical trials to clarify the influence of casein-derived MKP intake on baPWV.

The major strengths of this study are the well-controlled trial design and a sample size sufficient to detect a relatively small but clinically significant change in SBP due to casein-derived MKP intake. However, this study has several limitations. First, although significant difference in the change of SBP from baseline value between the groups was observed in this trial, there were no significant differences in the measured values. The reason for this could be that the effect of MKP on BP was smaller than expected. Indeed, the effect size of casein-derived MKP intake on SBP at week 12 was estimated to be 0.6, whereas the actual effect size was 0.26 (Table 2-3). Furthermore, the results of the subgroup analysis showed that the effect of MKP was greater in participants with high-normal BP than with grade 1 hypertension. Indeed, the results of subgroup analysis in the participants with high-normal BP showed that there was a significant improvement in the MKP group compared to the placebo group, not only in the change of SBP, but also in the mean DBP values (Table 2-4 and Figure 2-3). Therefore, it may be necessary to conduct a trial with a larger sample size of participants with high-normal BP to more accurately confirm the effect of MKP on BP. Second, the intervention period was only 12 weeks and tested only one casein-derived MKP dose. It is therefore necessary to conduct a longer-duration and multi-dose intervention to detect the effects of MKP over a more long-term period. Third, most studies examining the relationship between BP and CVD have been based

on seated office BP; however, 24-hour ambulatory BP is more useful than seated office BP for predicting CVD risk [36,37]. Similarly, although baPWV has been shown to be a good predictor of CVD [38], the gold standard in this field is carotid-femoral pulse wave velocity (cfPWV). Therefore, it is important to evaluate the effect of casein-derived MKP ingestion on 24-hour ambulatory BP and cfPWV.

2.5 Conclusions

The BP-lowering efficacy of daily casein-derived MKP ingestion was evaluated in participants with high-normal BP or grade 1 hypertension by a randomized, double-blind, placebo-controlled, parallel-group trial. The MKP group showed a clinically significant reduction in SBP but no change in baPWV. No serious AEs attributable to ingestion of the study products were recorded. Although it may be necessary to conduct a further clinical trial to more accurately confirm the effect of MKP on BP, regular casein-derived MKP ingestion has the potential to be a safe and effective SBP-lowering treatment for people with high-normal BP or grade 1 hypertension. Thus, regular casein-derived MKP ingestion could help decrease the risks of diseases associated with hypertension.

References

[1] Shimamoto K, Ando K, Fujita T, et al. The Japanese Society of Hypertension Guidelines for the Management of Hypertension (JSH 2014). *Hypertens Res*. 2014;37(4):253–390. doi:10.1038/hr.2014.20

[2] Kearney PM, Whelton M, Reynolds K, Muntner P, Whelton PK, He J. Global burden of hypertension: analysis of worldwide data. *Lancet*. 2005;365(9455):217–223. doi:10.1016/S0140-6736(05)17741-1

[3] Krousel-Wood MA, Muntner P, He J, Whelton PK. Primary prevention of essential hypertension. *Med Clin North Am*. 2004;88(1):223–238. doi:10.1016/s0025-7125(03)00126-3

[4] Massey LK. Dairy food consumption, blood pressure and stroke. *J Nutr*. 2001;131(7):1875–1878. doi:10.1093/jn/131.7.1875

[5] Appel LJ, Moore TJ, Obarzanek E, et al. A clinical trial of the effects of dietary patterns on blood pressure. DASH Collaborative Research Group. *N Engl J Med*. 1997;336(16):1117–1124. doi:10.1056/NEJM199704173361601

[6] Engberink MF, Hendriksen MA, Schouten EG, et al. Inverse association between dairy intake and hypertension: the Rotterdam Study. *Am J Clin Nutr*. 2009;89(6):1877–1883. doi:10.3945/ajcn.2008.27064

[7] Martínez-Maqueda D, Miralles B, Recio I, Hernández-Ledesma B. Antihypertensive peptides from food proteins: a review. *Food Funct*. 2012;3(4):350–361. doi:10.1039/c2fo10192k

[8] Xu JY, Qin LQ, Wang PY, Li W, Chang C. Effect of milk tripeptides on blood pressure: a meta-analysis of randomized controlled trials. *Nutrition*. 2008;24(10):933–940. doi:10.1016/j.nut.2008.04.004

[9] Boelsma E, Kloek J. Lactotripeptides and antihypertensive effects: a critical review. *Br J Nutr*. 2009;101(6):776–786. doi:10.1017/S0007114508137722

[10] Geleijnse JM, Engberink MF. Lactopeptides and human blood pressure. *Curr Opin Lipidol*. 2010;21(1):58–63. doi:10.1097/MOL.0b013e3283333813

[11] Cicero AF, Gerocarni B, Laghi L, Borghi C. Blood pressure lowering effect of lactotripeptides assumed as functional foods: a meta-analysis of current available clinical trials. *J Hum Hypertens*. 2011;25(7):425–436. doi:10.1038/jhh.2010.85

[12] Townsend RR, McFadden CB, Ford V, Cadée JA. A randomized, double-blind, placebo-controlled trial of casein protein hydrolysate (C12 peptide) in human essential hypertension. *Am J Hypertens*. 2004;17(11 Pt 1):1056–1058. doi:10.1016/j.amjhyper.2004.06.018

[13] Cadée JA, Chang CY, Chen CW, Huang CN, Chen SL, Wang CK. Bovine casein hydrolysate (C12 Peptide) reduces blood pressure in prehypertensive subjects. *Am J Hypertens*. 2007;20(1):1–5. doi:10.1016/j.amjhyper.2006.06.005

[14] FitzGerald RJ, Murray BA, Walsh DJ. Hypotensive peptides from milk proteins. *J Nutr*. 2004;134(4):980S–988S. doi:10.1093/jn/134.4.980S

[15] López-Fandiño R, Otte J, Van Camp J. Physiological, chemical and technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity. *Int Dairy J*. 2006;16(11):1277–1293. doi:10.1016/j.idairyj.2006.06.004

[16] Yamada A, Sakurai T, Ochi D, Mitsuyama E, Yamauchi K, Abe F. Novel angiotensin I-converting enzyme inhibitory peptide derived from bovine casein. *Food Chem*. 2013;141(4):3781–3789. doi:10.1016/j.foodchem.2013.06.089

[17] Yamada A, Sakurai T, Ochi D, Mitsuyama E, Yamauchi K, Abe F. Antihypertensive effect of the bovine casein-derived peptide Met-Lys-Pro. *Food Chem*. 2015;172:441–446. doi:10.1016/j.foodchem.2014.09.098

[18] Ochi D, Yamada A, Yamauchi K, Abe F, Tho Y. Blood pressure-lowering effects of casein-derived peptide Met-Lys-Pro in subjects with high-normal blood pressure and grade 1 hypertension –A randomized, double-blind, placebo-controlled, parallel-group, trial–. *Jpn Pharmacol Ther*. 2017;45(10):1637–1648.

[19] Laurent S, Boutouyrie P, Asmar R, et al. Aortic stiffness is an independent predictor of all-cause and cardiovascular mortality in hypertensive patients. *Hypertension*. 2001;37(5):1236–1241. doi:10.1161/01.hyp.37.5.1236

[20] Sakuragi S, Abhayaratna WP. Arterial stiffness: methods of measurement, physiologic determinants and prediction of cardiovascular outcomes. *Int J Cardiol*. 2010;138(2):112–118. doi:10.1016/j.ijcard.2009.04.027

[21] Miura K, Nakagawa H, Ohashi Y, et al. Four blood pressure indexes and the risk of stroke and myocardial infarction in Japanese men and women: a meta-analysis of 16 cohort studies. *Circulation*. 2009;119(14):1892–1898. doi:10.1161/CIRCULATIONAHA.108.823112

[22] Lewington S, Clarke R, Qizilbash N, Peto R, Collins R; Prospective Studies Collaboration. Age-specific relevance of usual blood pressure to vascular mortality: a meta-analysis of

individual data for one million adults in 61 prospective studies. *Lancet*. 2002;360(9349):1903–1913. doi:10.1016/s0140-6736(02)11911-8

[23] Matsui T. Are Peptides Absorbable Compounds? *J Agric Food Chem*. 2018;66(2):393–394. doi:10.1021/acs.jafc.7b05589

[24] Qingbiao Xu, Hui Hong, Jianping Wu, Xianghua Yan. Bioavailability of bioactive peptides derived from food proteins across the intestinal epithelial membrane: A review. *Trends in Food Sci Technol*. 2019;86:399–411. doi.org/10.1016/j.tifs.2019.02.050

[25] Dasgupta C, Zhang L. Angiotensin II receptors and drug discovery in cardiovascular disease. *Drug Discov Today*. 2011;16(1–2):22–34. doi:10.1016/j.drudis.2010.11.016

[26] Izzo JL Jr, Weir MR. Angiotensin-converting enzyme inhibitors. *J Clin Hypertens (Greenwich)*. 2011;13(9):667–675. doi:10.1111/j.1751-7176.2011.00508.x

[27] Nakashima EM, Kudo A, Iwaihara Y, Tanaka M, Matsumoto K, Matsui T. Application of ^{13}C stable isotope labeling liquid chromatography–multiple reaction monitoring–tandem mass spectrometry method for determining intact absorption of bioactive dipeptides in rats. *Anal Biochem*. 2011;414(1):109–116. doi:10.1016/j.ab.2011.02.037

[28] Matsui T, Tamaya K, Seki E, Osajima K, Matsumoto K, Kawasaki T. Val-Tyr as a natural antihypertensive dipeptide can be absorbed into the human circulatory blood system. *Clin Exp Pharmacol Physiol*. 2002;29(3):204–208. doi:10.1046/j.1440-1681.2002.03628.x

[29] Matsui T, Tamaya K, Seki E, Osajima K, Matsumo K, Kawasaki T. Absorption of Val-Tyr with *in vitro* angiotensin I-converting enzyme inhibitory activity into the circulating blood system of mild hypertensive subjects. *Biol Pharm Bull*. 2002;25(9):1228–1230. doi:10.1248/bpb.25.1228

[30] Matsui T, Imamura M, Oka H, et al. Tissue distribution of antihypertensive dipeptide, Val-Tyr, after its single oral administration to spontaneously hypertensive rats. *J Pept Sci*. 2004;10(9):535–545. doi:10.1002/psc.568

[31] Min LJ, Kobayashi Y, Mogi M, et al. Administration of bovine casein-derived peptide prevents cognitive decline in Alzheimer disease model mice. *PLoS One*. 2017;12(2):e0171515. doi:10.1371/journal.pone.0171515

[32] Yamashina A, Tomiyama H, Takeda K, et al. Validity, reproducibility, and clinical significance of noninvasive brachial-ankle pulse wave velocity measurement. *Hypertens Res*. 2002;25(3):359–364. doi:10.1291/hypres.25.359

[33] Tomiyama H, Yamashina A, Arai T, et al. Influences of age and gender on results of noninvasive brachial-ankle pulse wave velocity measurement—a survey of 12517 subjects. *Atherosclerosis*. 2003;166(2):303–309. doi:10.1016/s0021-9150(02)00332-5

[34] Ohnishi H, Saitoh S, Takagi S, et al. Pulse wave velocity as an indicator of atherosclerosis in impaired fasting glucose: the Tanno and Sobetsu study. *Diabetes Care*. 2003;26(2):437–440. doi:10.2337/diacare.26.2.437

[35] Munakata M, Konno S, Miura Y, Yoshinaga K; J-TOPP Study Group. Prognostic significance of the brachial-ankle pulse wave velocity in patients with essential hypertension: final results of the J-TOPP study. *Hypertens Res*. 2012;35(8):839–842. doi:10.1038/hr.2012.53

[36] Ohkubo T, Kikuya M, Metoki H, et al. Prognosis of "masked" hypertension and "white-coat" hypertension detected by 24-h ambulatory blood pressure monitoring 10-year follow-

up from the Ohasama study. *J Am Coll Cardiol*. 2005;46(3):508–515. doi:10.1016/j.jacc.2005.03.070

[37] Kikuya M, Ohkubo T, Asayama K, et al. Ambulatory blood pressure and 10-year risk of cardiovascular and noncardiovascular mortality: the Ohasama study. *Hypertension*. 2005;45(2):240–245. doi:10.1161/01.HYP.0000152079.04553.2c

[38] Van Bortel LM, Laurent S, Boutouyrie P, et al. Expert consensus document on the measurement of aortic stiffness in daily practice using carotid-femoral pulse wave velocity. *J Hypertens*. 2012;30(3):445-448. doi:10.1097/HJH.0b013e32834fa8b0

Chapter 3

Effect of Casein-Derived Met-Lys-Pro on Cognitive Function in Community-Dwelling Adults Without Dementia: A Randomized Controlled Trial

3.1 Introduction

As global life expectancy rises, the growth in the number of older adults with dementia is unprecedented. The number of individuals living with dementia is predicted to increase from 47 million in 2015 to 132 million by 2050 [1]. AD is the most common cause of dementia. Cholinesterase inhibitors and *N*-methyl-D-aspartate receptor antagonists have been approved for the treatment of AD in many countries [2]. Although these drugs provide a moderate treatment effect, they do not completely alter the condition [3,4]. Prevention and care for AD have become urgent global issues. AD progresses for many years before symptoms appear; and when symptoms become clinically apparent, the condition is already too advanced for treatment [5,6]. Therefore, taking preventative measures before the onset of clinical symptoms is highly recommended [7–9].

Recently, it was suggested that centrally active ACE inhibitors and angiotensin receptor blockers lower AD risk or slow its progression, independent of their BP-lowering effect [10,11]. The activity of ACE is elevated in the brains of patients with AD [12]. Ang II generated by ACE may promote oxidative stress and neuroinflammation, leading to neurodegeneration and brain aging [13,14]. However, several clinical studies have shown that centrally active ACE inhibitors, which cross the blood–brain barrier, prevent the process of neurodegeneration leading to dementia and the incidence of AD [11,15–17].

MKP has been identified as an anti-hypertensive tripeptide from bovine casein hydrolysate [18]. MKP exhibited strong ACE inhibitory activity *in vitro* (IC_{50} = 0.43 μM), and orally administered MKP was absorbed intact into the bloodstream and reduced BP in SHRs [19]. Moreover, the autoradiography data showed that MKP or its metabolites could be

distributed in the brain after the oral administration of ^{14}C-MKP [20]. Based on these results, its potential effects on cognitive function were examined. The results showed that oral administration of casein-derived MKP significantly attenuated cognitive decline in a mouse model of AD [20]. Therefore, casein-derived MKP intake has the potential to improve cognitive function in humans.

Improving cognitive function by the daily ingestion of effective food ingredients at a pre-AD stage may either prevent or delay the onset of AD [21]. Thus, a 24-week, randomized, double-blind, placebo-controlled trial was conducted to investigate the ability of casein-derived MKP to improve cognitive function in a population of community-dwelling adults without dementia. Since they represent a population at risk of developing clinical AD, but constitute a group in whom the preclinical disease is believed to be at an early enough stage to still respond to intervention, middle-aged and elderly individuals without dementia were recruited for the study [6]. The scores of the Japanese version of the AD Assessment Scale-cognitive subscale (ADAS-cog) [22,23] were used as the primary outcome. The ADAS-cog is one of the most frequently used instruments to evaluate general cognitive function in clinical trials. To the best of my knowledge, this is the first study to evaluate the effects of casein-derived MKP on human cognition. Therefore, the ADAS-cog was used in this study to broadly examine the effects of casein-derived MKP on cognitive function. As secondary outcomes, the revised version of Hasegawa's Dementia Scale (HDS-R) [24], the Japanese version of the Montreal Cognitive Assessment (MoCA-J) [25,26], and the eight-item Short-Form Health Survey (SF-8) [27] were used. The safety of casein-derived MKP was also evaluated.

3.2 Methods

3.2.1 Participants

Adult volunteers living in Matsumoto, Japan and the surrounding areas were recruited through website announcements, advertisements, and mailed invitations. Recruitment was conducted from April to June 2018. Inclusion criteria included: age ≥ 40 years and an HDS-R score of 21–30. Exclusion criteria included: history or presence of dementia; suspected dementia; mental disorders such as schizophrenia and depression; serious diseases of the brain, liver, kidneys, heart, lungs, gastrointestinal tract, blood, or metabolism; serious allergies to medicine or food; pregnancy, lactation, or planning to get pregnant during the study period; ineligibility due to physician's diagnosis based on participant background, physical examination, and interview.

The study protocol was examined and approved by the institutional review board and the Ethics Committee of Matsumoto Junior College (approval code: 201704). The study was conducted in accordance with the Declaration of Helsinki and the Ethical Guidelines for Medical and Health Research Involving Human Subjects (Ministry of Education, Culture, Sports, Science and Technology; Ministry of Health, Labor and Welfare, Japan). After a detailed explanation regarding the study objectives and procedures, all participants provided written informed consent. They were informed that they were free to withdraw at any time without obligation. This trial was registered at the UMIN-CTR as UMIN000032833.

3.2.2 Study Products

All study products were delivered in tablet form. The composition of the study products is detailed in Table 3-1. The MKP-containing tablets were made from casein hydrolysate manufactured by the Morinaga Milk Industry (Tokyo, Japan) [19]. Each tablet contained 50 μg MKP in 0.25 g casein hydrolysate. The placebo tablets contained 0.25 g dextrin with no detectable MKP content. MKP and placebo tablets were matched for appearance.

Table 3-1 Composition of the study products.

Component	MKP (4 tablets/day)	Placebo (4 tablets/day)
Energy (kcal)	7.0	7.0
Protein (g)	0.9	0.0
Fat (g)	0.0	0.0
Carbohydrates (g)	0.9	1.8
Sodium (mg)	11.0	0.0
Casein hydrolysate containing MKP (g/day)	1.0	0.0
MKP (μg/day)	200.0	0.0
Dextrin (g/day)	0.0	1.0

3.2.3 Procedures

The trial was conducted in Matsumoto between June 2018 and February 2019. To investigate the impact of regular casein-derived MKP intake on cognitive function in community-dwelling adults without dementia, a 24-week randomized, double-blind, placebo-controlled trial was conducted. The study lasted 24 weeks and 2 weeks for treatment and post-treatment observation,

respectively. Efficacy assessments were obtained at baseline, at week 12, and at week 24.

Eligibility was assessed based on interviews, physical examination (BP, body height, BW), self-reported data from health and lifestyle questionnaires, the Geriatric Depression Scale (GDS) [28,29] score, and the HDS-R score. Individuals with scores ≥ 7 on the GDS or ≤ 20 on the HDS-R were excluded from participation. Eligible participants were randomly assigned to receive MKP or placebo tablets in a 1:1 ratio by a person not directly involved in the study using computer-generated lists of random numbers via the randomly permuted block method. The participants in the MKP and placebo groups were instructed to consume four tablets daily at their leisure, respectively. The participants, physician, researchers assessing outcomes, and researchers conducting statistical analyses were blinded to the treatment group allocation over the study duration.

All participants were encouraged to continue with their usual daily activities and diet throughout the study period. The participants were also asked to keep a daily record of their experiences and occurrences. These included items related to supplementation of study products, illness, use of medications or other nutritional supplements, and hospital visits. Study staff interviewed participants before and throughout the study to ensure their compliance with these lifestyle requirements based on the participant diaries. Treatment compliance was assessed by counting the number of tablets returned at the time of the final study visit and the inspection of the participant diaries.

3.2.4 Outcome Measurements

The primary outcome measure was the ADAS-cog score [22,23]. The test, which yields a score ranging from 0 (no errors) to 70 (maximum impairment), assesses memory, language, praxis, and orientation and is composed of 11 subscales (word recall, spoken language ability, comprehension of spoken language, word-finding difficulty, following commands, naming objects and fingers, constructions, ideational praxis, orientation, word recognition, and recall of test instructions) to evaluate general cognitive function.

The secondary outcome measures were the HDS-R, MoCA-J, and SF-8 scores. In Japan, the HDS-R is a neuropsychological battery commonly used for the screening of dementia [24]. Individuals with scores of ≤ 20 out of 30 are diagnosed with suspected dementia. The MoCA-J is a useful screening tool for detecting mild cognitive impairment (MCI) [25,26]. The cut-off point of the test for MCI and AD is 25/26 out of 30. To correct for the educational background, one point is added for participants with a total score < 30 and an educational background of < 12 years. The ADAS-cog and MoCA-J were performed at baseline and after 12 and 24 weeks of supplementation. The HDS-R was performed at baseline and after 24 weeks of supplementation. The participants self-reported assessment of physical and mental health at baseline and week 24 was obtained using the SF-8 [27]. The Mental health Component Summary (MCS) and Physical health Component Summary (PCS) scores were calculated.

3.2.5 Safety Monitoring

All intervened participants were monitored throughout the study for AEs and side-effects. Safety monitoring comprised of a questionnaire that assessed general health and the occurrence

of any health-related events. The relation of AEs to ingestion of the study products was determined by the physician while remaining blinded to group allocation. The severity of AEs was evaluated according to the CTCAE version 4.0 JCOG/JSCO.

3.2.6 Sample Size

The effect size Cohen's *d* of the ADAS-cog total score at 24 weeks after casein-derived MKP intake was estimated to be 0.40. The sample size required to detect a mean ADAS-cog total score difference at $\alpha = 0.05$ and power = 0.90 by unpaired *t*-test was calculated at 133 study participants per group, making up a total of 266 study participants. The target sample size was calculated using G*Power 3.1.9.2 (Heinrich Heine Universitat, Dusseldorf, Germany). Considering a 10% dropout rate, approximately 150 participants per group needed to be recruited.

3.2.7 Statistical Analysis

Statistical analysis was based on the intention-to-treat population, which included all randomly assigned participants with at least one observation. Missing data were handled by the available case analysis. Data were presented as mean along with SE, except for baseline values, which were expressed as mean along with SD. The baseline characteristics of the study groups were compared using Fisher's exact test for categorical variables and unpaired *t*-test for continuous variables. The continuous variables of efficacy were assessed by analysis of covariance models to adjust for baseline values, using week 24 values as the dependent variables. The cognitive test data were also analyzed according to age, MoCA-J score, or medication status by dividing

the participants into predefined subgroups. Age was divided by 65, the standard for elderly people in Japan. The MoCA-J score was divided by 26, the standard for suspected MCI. Medication status was divided by whether the participants were using regular medication; temporary medication, such as for colds, was not included. Safety analyses were carried out based on summary listings of AEs, with Fisher's exact test used for pairwise comparisons. All comparisons were two-tailed, and the statistical significance level was set to $P < 0.05$. All analyses were performed using IBM SPSS Statistics version 23 (IBM Corp., Armonk, NY, USA).

3.3 Results

3.3.1 Participants

From a total of 468 participants screened for the study, 268 were enrolled and randomly allocated into the MKP (n = 134) and placebo (n = 134) groups (Figure 3-1). Out of the 268 participants enrolled, 256 and 253 remained enrolled in the study for 12 weeks and until the end of the study period, respectively. Three randomized participants withdrew before the intervention for personal reasons unrelated to the trial and 12 (six in the MKP and the placebo group, respectively) discontinued; nine (six and three in the MKP and the placebo group, respectively) dropped out during the intervention period due to personal reasons unrelated to the trial, and three (all in the placebo group) due to AEs unrelated to the treatment. The overall dropout rate was 5.6% (15 of 268). The compliance rates were 96.7% and 96.5% in the MKP and placebo group, respectively, with the difference being non-significant. Table 3-2 shows the baseline characteristics, including sex, age, BMI, BP, education years, SF-8, GDS, ADAS-cog,

MoCA-J, and HDS-R scores. The two groups did not differ significantly in the baseline demographic variables. In the overall population, the mean age was 68.3 years, the mean ADAS-cog score was 4.1, the mean MoCA-J score was 25.8, and the mean HDS-R score was 28.6. Considering the cut-off threshold of the MoCA-J score (25/26), 58% of all enrolled participants were considered cognitively healthy, and 42% were suspected to have MCI.

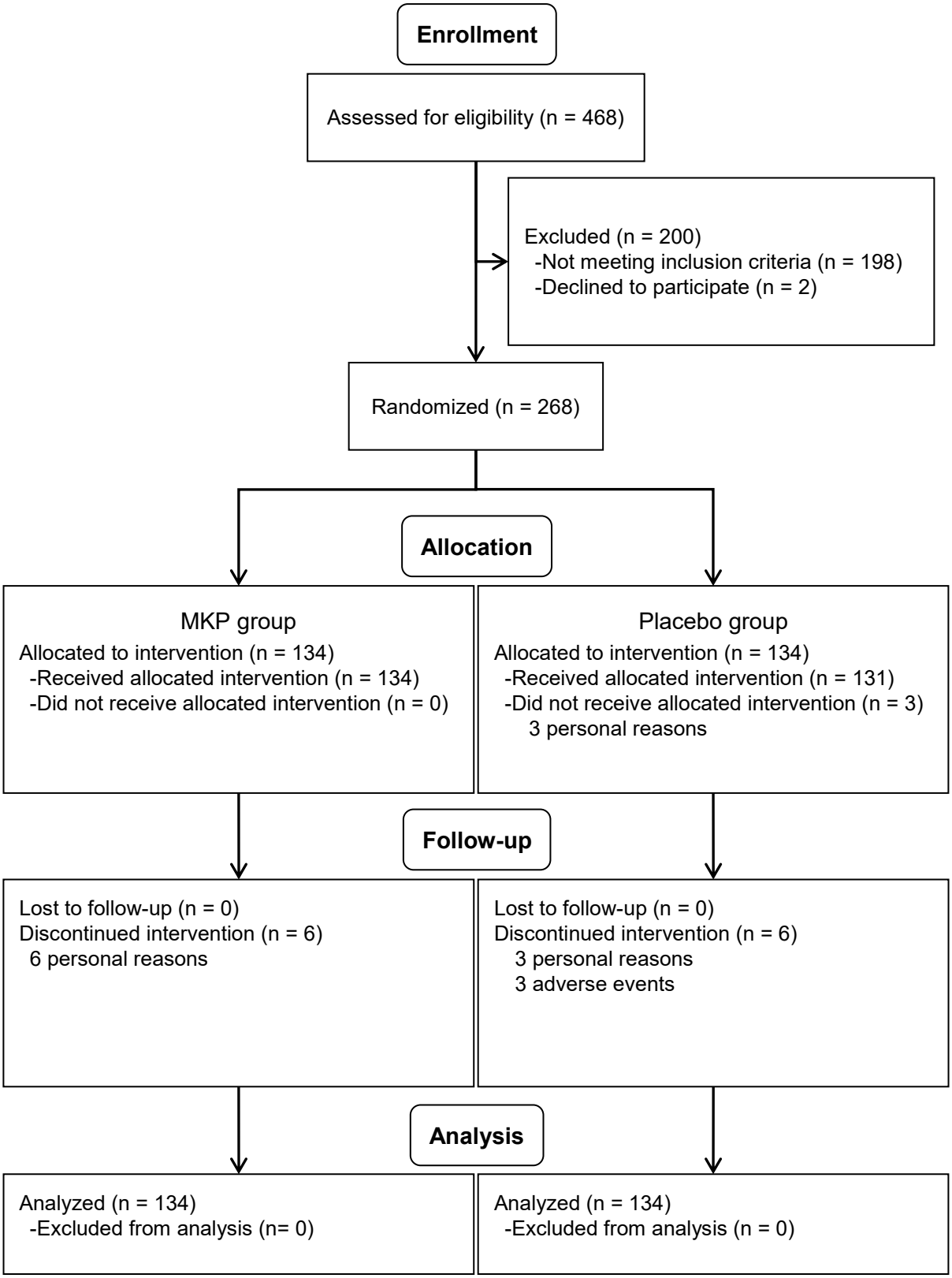

Figure 3-1 Study flow diagram.

Table 3-2 Baseline characteristics of the participants.

Characteristic	MKP (n = 134)	Placebo (n = 134)
Male/Female (n)	43/91	42/92
Age (years)	68.1 ± 8.4	68.5 ± 8.0
BMI (kg/m^2)	23.0 ± 2.9	22.5 ± 3.0
SBP (mmHg)	126.1 ± 13.2	127.2 ± 14.4
DBP (mmHg)	71.4 ± 9.4	73.5 ± 10.5
Education (years)	13.2 ± 1.7	13.0 ± 1.8
SF-8, MCS	51.8 ± 5.5	52.1 ± 4.7
SF-8, PCS	61.7 ± 8.1	62.3 ± 7.2
GDS	1.6 ± 1.6	1.5 ± 1.5
ADAS-cog	4.1 ± 2.2	4.1 ± 2.1
MoCA-J	25.8 ± 2.8	25.9 ± 2.9
HDS-R	28.7 ± 1.5	28.6 ± 1.3

Data represent numbers of participants or mean ± SD. SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; SF-8, eight-item Short-Form Health Survey; MCS, Mental health Component Summary; PCS, Physical health Component Summary; GDS, Geriatric Depression Scale; ADAS-cog, Alzheimer's Disease Assessment Scale-cognitive subscale; MoCA-J, Japanese version of the Montreal Cognitive Assessment; HDS-R, revised version of Hasegawa's Dementia Scale.

3.3.2 Outcomes

A summary of the cognitive test data at baseline and after 12 and 24 weeks is presented in Table 3-3. After 24 weeks, there was no significant casein-derived MKP treatment effect on the ADAS-cog total score compared to placebo. The orientation of the participants in the MKP group, as measured by the respective ADAS-cog subscale, significantly improved (P = 0.022, Cohen's d = 0.30). There were no significant differences between the groups in the other cognitive variables.

A subgroup analysis of age, MoCA-J score, and medication status were performed. The results of the analysis are shown in Tables 3-4, 3-5, and 3-6. The study of the subgroup of elderly participants (age ≥ 65 years) revealed a statistically significant treatment effect between the groups with regard to construction ($P = 0.049$, Cohen's $d = 0.28$) and orientation ($P = 0.039$, Cohen's $d = 0.34$), as measured by the respective subscales of the ADAS-cog (Table 3-4). There were no significant differences between the groups in terms of other cognitive variables in the subgroup analysis by age. The study of the subgroup of cognitively healthy participants (MoCA-J score ≥ 26) revealed a statistically significant treatment effect for orientation ($P = 0.029$, Cohen's $d = 0.37$) and HDS-R score ($P = 0.033$, Cohen's $d = 0.37$) between the groups (Table 3-5). There were no significant differences between the groups in terms of the other cognitive variables in the subgroup analysis by MoCA-J score. The analysis of the subgroup "without medication" revealed a statistically significant treatment effect for orientation ($P = 0.003$, Cohen's $d = 0.62$) between the groups (Table 3-6). There were no significant differences between the groups in terms of the other cognitive variables in the subgroup analysis by medication status. Table 3-7 presents the MCS and PCS values of SF-8 before and after daily intake of casein-derived MKP or placebo. There was no significant casein-derived MKP treatment effect on SF-8 compared to placebo.

Table 3-3 Summary of the cognitive tests.

	Group	Baseline	Week 12	Week 24	*P* value	ES *d*
Number of participants	M	134	129	128		
	P	134	127	125		
ADAS-cog total score	M	4.08 (0.19)	3.94 (0.19)	3.18 (0.17)	0.302	0.12
	P	4.10 (0.18)	4.21 (0.21)	3.43 (0.19)		
Word recall	M	2.33 (0.10)	2.69 (0.11)	1.85 (0.10)	0.635	0.08
	P	2.40 (0.11)	2.87 (0.13)	1.93 (0.10)		
Spoken language ability	M	0.01 (0.01)	0.00 (0.00)	0.00 (0.00)	NA	NA
	P	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)		
Comprehension of spoken language	M	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	NA	NA
	P	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)		
Word-finding difficulty	M	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	NA	NA
	P	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)		
Following commands	M	0.28 (0.04)	0.29 (0.05)	0.27 (0.04)	0.299	0.11
	P	0.31 (0.05)	0.23 (0.04)	0.22 (0.04)		
Naming	M	0.00 (0.00)	0.01 (0.01)	0.00 (0.00)	NA	NA
	P	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)		
Constructions	M	0.09 (0.04)	0.02 (0.01)	0.01 (0.01)	0.054	0.24
	P	0.10 (0.03)	0.03 (0.02)	0.08 (0.04)		
Ideational praxis	M	0.09 (0.04)	0.03 (0.02)	0.03 (0.02)	0.625	0.07
	P	0.04 (0.02)	0.09 (0.04)	0.02 (0.02)		
Orientation	M	0.10 (0.03)	0.08 (0.03)	0.05 (0.02)	0.022†	0.30
	P	0.13 (0.03)	0.11 (0.03)	0.15 (0.03)		
Word recognition	M	1.18 (0.09)	0.83 (0.08)	0.97 (0.08)	0.677	0.04
	P	1.13 (0.09)	0.88 (0.09)	1.01 (0.09)		
Recall of test instructions	M	0.01 (0.01)	0.00 (0.00)	0.00 (0.00)	0.313	0.13
	P	0.01 (0.01)	0.00 (0.00)	0.01 (0.01)		
MoCA-J	M	25.82 (0.24)	26.36 (0.23)	27.34 (0.20)	0.417	0.08
	P	25.87 (0.25)	26.18 (0.23)	27.14 (0.22)		
HDS-R	M	28.67 (0.13)		28.18 (0.18)	0.885	0.05
	P	28.57 (0.12)		28.08 (0.18)		

Data represent mean (with SE). †$P < 0.05$ (vs. placebo). *P* values were derived by the analysis of covariance (the scores at week 24 were adjusted for the baseline score). M, MKP; P, placebo; ES, effect size; NA, not applicable.; ADAS-cog, Alzheimer's Disease Assessment Scale-cognitive subscale; MoCA-J, Japanese version of the Montreal Cognitive Assessment; HDS-R, revised version of Hasegawa's Dementia Scale.

Table 3-4 Subgroup analysis of the cognitive tests by age.

	Group	Age < 65 years		Age ≥ 65 years	
		Baseline	Week 24	Baseline	Week 24
Number of participants	M	33	32	101	96
	P	35	32	99	93
ADAS-cog total score	M	3.35 (0.39)	2.88 (0.32)	4.32 (0.21)	3.28 (0.20)
	P	3.09 (0.28)	2.68 (0.24)	4.45 (0.21)	3.69 (0.23)
Word recall	M	1.81 (0.20)	1.75 (0.21)	2.50 (0.11)	1.88 (0.12)
	P	1.97 (0.16)	1.86 (0.16)	2.54 (0.13)	1.96 (0.12)
Spoken language ability	M	0.00 (0.00)	0.00 (0.00)	0.01 (0.01)	0.00 (0.00)
	P	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Comprehension of spoken	M	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
language	P	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Word-finding difficulty	M	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	P	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Following commands	M	0.30 (0.08)	0.31 (0.08)	0.28 (0.05)	0.26 (0.05)
	P	0.29 (0.10)	0.16 (0.07)	0.31 (0.05)	0.25 (0.04)
Naming	M	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	P	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Constructions	M	0.00 (0.00)	0.00 (0.00)	0.12 (0.05)	0.01 (0.01)†
	P	0.03 (0.03)	0.00 (0.00)	0.12 (0.03)	0.11 (0.05)
Ideational praxis	M	0.03 (0.03)	0.00 (0.00)	0.11 (0.05)	0.04 (0.03)
	P	0.00 (0.00)	0.00 (0.00)	0.05 (0.03)	0.02 (0.02)
Orientation	M	0.18 (0.08)	0.03 (0.03)	0.07 (0.03)	0.06 (0.03)†
	P	0.09 (0.06)	0.06 (0.04)	0.14 (0.04)	0.18 (0.04)
Word recognition	M	1.02 (0.21)	0.79 (0.18)	1.23 (0.10)	1.03 (0.09)
	P	0.69 (0.10)	0.60 (0.11)	1.28 (0.11)	1.16 (0.11)
Recall of test instructions	M	0.00 (0.00)	0.00 (0.00)	0.01 (0.01)	0.00 (0.00)
	P	0.03 (0.03)	0.00 (0.00)	0.00 (0.00)	0.01 (0.01)
MoCA-J	M	26.79 (0.37)	27.66 (0.34)	25.50 (0.30)	27.24 (0.24)
	P	27.09 (0.44)	28.25 (0.24)	25.44 (0.29)	26.76 (0.28)
HDS-R	M	28.79 (0.20)	29.28 (0.17)	28.63 (0.16)	27.81 (0.22)
	P	28.74 (0.18)	29.41 (0.19)	28.51 (0.14)	27.62 (0.21)

Data represent mean (with SE). †$P < 0.05$ (vs. placebo). *P* values were derived by the analysis of covariance (the scores at week 24 were adjusted for the baseline score). M, MKP; P, placebo; ADAS-cog, Alzheimer's Disease Assessment Scale-cognitive subscale; MoCA-J, Japanese version of the Montreal Cognitive Assessment; HDS-R, revised version of Hasegawa's Dementia Scale.

Table 3-5 Subgroup analysis of the cognitive tests by MoCA-J score.

	Group	MoCA-J < 26		MoCA-J ≥ 26	
		Baseline	Week 24	Baseline	Week 24
Number of participants	M	57	54	77	74
	P	55	52	79	73
ADAS-cog total score	M	5.25 (0.28)	4.15 (0.29)	3.22 (0.20)	2.48 (0.16)
	P	5.19 (0.26)	4.17 (0.29)	3.34 (0.21)	2.90 (0.23)
Word recall	M	2.93 (0.14)	2.44 (0.16)	1.89 (0.12)	1.41 (0.11)
	P	2.95 (0.15)	2.31 (0.16)	2.01 (0.13)	1.66 (0.12)
Spoken language ability	M	0.02 (0.02)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	P	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Comprehension of spoken	M	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
language	P	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Word-finding difficulty	M	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	P	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Following commands	M	0.39 (0.07)	0.30 (0.07)	0.21 (0.05)	0.26 (0.05)
	P	0.33 (0.07)	0.31 (0.06)	0.29 (0.06)	0.16 (0.04)
Naming	M	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	P	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Constructions	M	0.14 (0.05)	0.00 (0.00)	0.05 (0.05)	0.01 (0.01)
	P	0.15 (0.05)	0.13 (0.08)	0.06 (0.03)	0.04 (0.02)
Ideational praxis	M	0.14 (0.08)	0.02 (0.02)	0.05 (0.03)	0.04 (0.04)
	P	0.02 (0.02)	0.04 (0.04)	0.05 (0.04)	0.00 (0.00)
Orientation	M	0.19 (0.06)	0.11 (0.05)	0.03 (0.02)	0.01 (0.01)†
	P	0.29 (0.07)	0.23 (0.07)	0.01 (0.01)	0.10 (0.03)
Word recognition	M	1.43 (0.15)	1.29 (0.16)	0.99 (0.11)	0.74 (0.07)
	P	1.44 (0.14)	1.14 (0.12)	0.91 (0.10)	0.92 (0.13)
Recall of test instructions	M	0.02 (0.02)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	P	0.02 (0.02)	0.00 (0.00)	0.00 (0.00)	0.01 (0.01)
MoCA-J	M	23.09 (0.24)	26.13 (0.34)	27.84 (0.15)	28.23 (0.18)
	P	22.96 (0.26)	25.83 (0.39)	27.90 (0.14)	28.08 (0.20)
HDS-R	M	28.07 (0.23)	27.00 (0.33)	29.12 (0.13)	29.04 (0.13)†
	P	28.02 (0.21)	27.50 (0.31)	28.95 (0.12)	28.49 (0.20)

Data represent mean (with SE). †$P < 0.05$ (vs. placebo). *P* values were derived by the analysis of covariance (the scores at week 24 were adjusted for the baseline score). M, MKP; P, placebo; ADAS-cog, Alzheimer's Disease Assessment Scale-cognitive subscale; MoCA-J, Japanese version of the Montreal Cognitive Assessment; HDS-R, revised version of Hasegawa's Dementia Scale.

Table 3-6 Subgroup analysis of the cognitive tests by medication status.

	Group	Without medication		With medication	
		Baseline	Week 24	Baseline	Week 24
Number of participants	M	56	51	78	77
	P	52	43	82	82
ADAS-cog total score	M	4.01 (0.30)	3.05 (0.28)	4.13 (0.24)	3.27 (0.21)
	P	3.80 (0.31)	3.05 (0.30)	4.29 (0.22)	3.62 (0.24)
Word recall	M	2.29 (0.16)	1.75 (0.18)	2.36 (0.13)	1.91 (0.12)
	P	2.23 (0.16)	1.81 (0.17)	2.50 (0.14)	2.00 (0.13)
Spoken language ability	M	0.00 (0.00)	0.00 (0.00)	0.01 (0.01)	0.00 (0.00)
	P	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Comprehension of spoken	M	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
language	P	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Word-finding difficulty	M	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	P	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Following commands	M	0.29 (0.07)	0.25 (0.07)	0.28 (0.05)	0.29 (0.05)
	P	0.25 (0.08)	0.16 (0.06)	0.34 (0.06)	0.26 (0.05)
Naming	M	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	P	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Constructions	M	0.13 (0.08)	0.00 (0.00)	0.06 (0.03)	0.01 (0.01)
	P	0.12 (0.04)	0.05 (0.03)	0.09 (0.03)	0.10 (0.05)
Ideational praxis	M	0.11 (0.06)	0.02 (0.02)	0.08 (0.04)	0.04 (0.04)
	P	0.00 (0.00)	0.00 (0.00)	0.06 (0.04)	0.02 (0.02)
Orientation	M	0.07 (0.03)	0.00 (0.00)††	0.12 (0.04)	0.09 (0.04)
	P	0.13 (0.06)	0.16 (0.06)	0.12 (0.04)	0.15 (0.04)
Word recognition	M	1.13 (0.15)	1.03 (0.15)	1.21 (0.12)	0.93 (0.10)
	P	1.05 (0.14)	0.85 (0.14)	1.18 (0.11)	1.10 (0.12)
Recall of test instructions	M	0.00 (0.00)	0.00 (0.00)	0.01 (0.01)	0.00 (0.00)
	P	0.02 (0.02)	0.02 (0.02)	0.00 (0.00)	0.00 (0.00)
MoCA-J	M	26.55 (0.32)	27.59 (0.33)	25.29 (0.34)	27.18 (0.25)
	P	26.79 (0.35)	27.65 (0.31)	25.29 (0.33)	26.88 (0.30)
HDS-R	M	28.64 (0.18)	28.37 (0.28)	28.69 (0.18)	28.05 (0.24)
	P	28.79 (0.18)	28.47 (0.29)	28.43 (0.15)	27.88 (0.22)

Data represent mean (with SE). ††$P < 0.005$ (vs. placebo). *P* values were derived by the analysis of covariance (the scores at week 24 were adjusted for the baseline score). M, MKP; P, placebo; ADAS-cog, Alzheimer Disease's Assessment Scale-cognitive subscale; MoCA-J, Japanese version of the Montreal Cognitive Assessment; HDS-R, revised version of Hasegawa's Dementia Scale.

Table 3-7 Summary of the SF-8.

	Group	Baseline	Week 24	*P* value
Number of participants	M	134	128	
	P	134	125	
SF-8, MCS	M	51.90 ± 0.44	50.92 ± 0.47	0.233
	P	52.01 ± 0.41	50.48 ± 0.50	
SF-8, PCS	M	61.97 ± 0.66	62.90 ± 0.72	0.263
	P	62.31 ± 0.62	62.41 ± 0.73	

Data represent mean ± SE. *P* values were derived by the analysis of covariance (the scores at week 24 were adjusted for the baseline score). SF-8, eight-item Short-Form Health Survey; M, MKP; P, placebo; MCS, Mental health Component Summary; PCS, Physical health Component Summary.

3.3.3 Safety

As shown in Table 3-8, there were no significant differences between the groups in the incidence of AEs during the 24 weeks of treatment and 2 weeks of post-treatment observation. In total, 306 AEs were reported by 243 participants throughout the study; 145 were reported by 114 participants in the MKP group and 161 by 129 participants in the placebo group. Upper respiratory infection was the most common AE (32.1% in the MKP vs. 33.6% in the placebo group, $P = 0.896$). No AE was related to the study products.

Table 3-8 Intervened participants with AEs by system organ class.

System Organ Class	MKP (n = 134) n (%)	Placebo (n = 131) n (%)	*P* value
Infections and infestations	52 (38.8)	54 (41.2)	0.708
Nervous system disorders	16 (11.9)	14 (10.7)	0.847
Gastrointestinal disorders	13 (9.7)	15 (11.5)	0.693
Musculoskeletal and connective tissue disorders	7 (5.2)	9 (6.9)	0.615
Respiratory, thoracic and mediastinal disorders	5 (3.7)	8 (6.1)	0.408
General disorders and administration site conditions	2 (1.5)	1 (0.8)	1.000
Injury, poisoning and procedural complications	2 (1.5)	2 (1.5)	1.000
Cardiac disorders	1 (0.7)	1 (0.8)	1.000
Immune system disorders	1 (0.7)	2 (1.5)	0.619
Neoplasms benign, malignant and unspecified	1 (0.7)	2 (1.5)	0.619
Renal and urinary disorders	1 (0.7)	0 (0.0)	1.000
Reproductive system and breast disorders	1 (0.7)	0 (0.0)	1.000
Skin and subcutaneous tissue disorders	1 (0.7)	5 (3.8)	0.117
Ear and labyrinth disorders	0 (0.0)	1 (0.8)	0.494

P values were derived by Fisher's exact test. AE, adverse event.

3.4 Discussion

In recent years, the search for the best strategy to reduce AD incidence and prevalence in cognitively healthy individuals at risk of developing AD has attracted marked attention [8]. In fact, a considerable body of epidemiological evidence supports that modifiable lifestyle-related factors are associated with the development of pre-dementia and dementia syndromes in later life [30]. Furthermore, several healthy dietary plans and food compositions have preventive effects on cognitive decline [21,31–33]. The present study showed that the casein-derived MKP supplementation may have the potential to improve orientation in community-dwelling adults

without dementia, with good tolerability, and no treatment-related AEs, during 24 weeks of treatment and 2 weeks post-treatment. In addition, although these data were exploratory, the results of the pre-specified subgroup analyses suggested that the benefits of casein-derived MKP may be more likely to appear in elderly individuals aged ≥ 65 years, those with healthy cognitive function, and those who do not use regular medications. Therefore, the results of this trial suggested that casein-derived MKP may be effective in treating individuals in the preclinical stage of AD or dementia, especially elderly people who are not suffering from any disease. Orientation is the ability to correctly identify one's own location in space and time, and serves as a useful indicator of cognitive decline [34]. Therefore, systematic dietary supplementation with casein-derived MKP may be a promising intervention toward safe and improved orientation before the onset of AD or dementia.

It was described that casein-derived MKP improved on cognitive function in an AD mouse model induced by ICV injection of Aβ42 using the Morris water maze [20]. In addition, the hippocampus was collected after behavioral testing, and inflammatory cytokine and nicotinamide adenine dinucleotide phosphate oxidase subunit expression was measured [20]. Consequently, daily administration of casein-derived MKP markedly attenuated Aβ42-induced cognitive decline and reduced Aβ42-induced tumor necrosis factor-α, monocyte chemoattractant protein-1, inducible nitric oxide synthase, $p47^{phox}$, and $gp91^{phox}$ expression. Hippocampus plays central roles in spatial and temporal processing [35]. Furthermore, a clinicopathological study of patients with AD found that spatial and temporal disorientation were associated with neurofibrillary tangle densities in the Brodmann areas 7 and 23, and the CA1 field of the hippocampus [36]. These findings suggest that casein-derived MKP may

improve orientation by supporting hippocampal function.

Evaluation of the bioavailability is important for understanding the physiological functions of bioactive peptides. Recently, it was reported that dipeptide Tyr-Pro could pass through the blood–brain barrier in an intact form and accumulate in the hippocampus, hypothalamus, striatum, cerebral cortex, and cerebellum of mouse brain [37]. Besides, orally administered Tyr-Pro significantly improved memory impairment in Aβ-injected AD model mice [38]. Considering these advanced evidence, further studies on MKP are needed to clarify its bioavailability in the brain as well as underlying mechanisms.

In this study, the ADAS-cog scores were used as the primary endpoint. Although ADAS-cog is the gold standard for confirming general cognitive function in AD trials, it was suggested to be less sensitive in individuals with normal cognitive function and MCI [39]. Indeed, the effect size of the casein-derived MKP intake on the ADAS-cog total score was estimated to be 0.40, whereas the actual effect size was 0.12 (Table 3-3). On the other hand, orientation, as measured by an ADAS-cog subscale, was reported to be an independently sensitive test item for people with MCI and mild AD [40]. Therefore, it may be necessary to use more sensitive neuropsychological tests for cognitively healthy individuals and individuals with MCI to investigate the impact of casein-derived MKP on cognitive function in more detail.

In Chapter 2, it was reported that regular casein-derived MKP intake was a safe and effective SBP-lowering treatment for individuals with high-normal BP or grade 1 hypertension in a clinical trial [41]. In this study, BP was measured only at baseline. Thus, the relationship between the casein-derived MKP effect on orientation and BP was not elucidated. Alternatively, other tests using the AD mouse model showed that casein-derived MKP intake did not affect

normal BP [20]. Besides, BP before intervention in the present participants was not high (Table 3-2). Therefore, these results may be independent of the effect of casein-derived MKP on BP. In the future, to clarify this mechanism of action, it will be crucial to examine how casein-derived MKP intake affects BP and cognitive function.

This study had several further limitations. First, considering the effects observed in this trial, the sample size might have been too small to fully evaluate the effect of casein-derived MKP intake on cognitive function in adults without dementia. In addition, the intervention period was limited to 24 weeks and a single casein-derived MKP-dose protocol. Furthermore, the casein-derived MKP impact on patients with manifested dementia remains unknown. It may be necessary to conduct a larger-scale, longer-duration, and multi-dose intervention to detect the casein-derived MKP effects on adults with and without dementia.

3.5 Conclusions

This study evaluated the impact of casein-derived MKP on non-demented community-dwelling adults by a randomized, double-blind, placebo-controlled trial. The results of the present study suggested the safety of daily casein-derived MKP intake and its potential to improve orientation in adults without dementia. However, this study was exploratory. Therefore, further studies are warranted to confirm these findings and the beneficial effects of casein-derived MKP on cognitive function.

References

[1] World Health Organization. Global action plan on the public health response to dementia 2017–2025. http://www.who.int/mental_health/neurology/dementia/action_plan_2017_2025/en/. Accessed February 21, 2021.

[2] Hansen RA, Gartlehner G, Webb AP, Morgan LC, Moore CG, Jonas DE. Efficacy and safety of donepezil, galantamine, and rivastigmine for the treatment of Alzheimer's disease: a systematic review and meta-analysis. *Clin Interv Aging*. 2008;3(2):211–225.

[3] Anand R, Gill KD, Mahdi AA. Therapeutics of Alzheimer's disease: Past, present and future. *Neuropharmacology*. 2014;76 Pt A:27–50. doi:10.1016/j.neuropharm.2013.07.004

[4] Schneider LS, Mangialasche F, Andreasen N, et al. Clinical trials and late-stage drug development for Alzheimer's disease: an appraisal from 1984 to 2014. *J Intern Med*. 2014;275(3):251–283. doi:10.1111/joim.12191

[5] Jack CR Jr, Knopman DS, Jagust WJ, et al. Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. *Lancet Neurol*. 2010;9(1):119–128. doi:10.1016/S1474-4422(09)70299-6

[6] Sperling RA, Aisen PS, Beckett LA, et al. Toward defining the preclinical stages of Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement*. 2011;7(3):280–292. doi:10.1016/j.jalz.2011.03.003

[7] McDade E, Bateman RJ. Stop Alzheimer's before it starts. *Nature*. 2017;547(7662):153–155. doi:10.1038/547153a

[8] Crous-Bou M, Minguillón C, Gramunt N, Molinuevo JL. Alzheimer's disease prevention: from risk factors to early intervention. *Alzheimers Res Ther*. 2017;9(1):71. doi:10.1186/s13195-017-0297-z

[9] Livingston G, Sommerlad A, Orgeta V, et al. Dementia prevention, intervention, and care. *Lancet*. 2017;390(10113):2673–2734. doi:10.1016/S0140-6736(17)31363-6

[10] Davies NM, Kehoe PG, Ben-Shlomo Y, Martin RM. Associations of anti-hypertensive treatments with Alzheimer's disease, vascular dementia, and other dementias. *J Alzheimers Dis*. 2011;26(4):699–708. doi:10.3233/JAD-2011-110347

[11] O'Caoimh R, Healy L, Gao Y, et al. Effects of centrally acting angiotensin converting enzyme inhibitors on functional decline in patients with Alzheimer's disease. *J Alzheimers Dis*. 2014;40(3):595–603. doi:10.3233/JAD-131694

[12] Miners JS, Ashby E, Van Helmond Z, et al. Angiotensin-converting enzyme (ACE) levels and activity in Alzheimer's disease, and relationship of perivascular ACE-1 to cerebral amyloid angiopathy. *Neuropathol Appl Neurobiol*. 2008;34(2):181–193. doi:10.1111/j.1365-2990.2007.00885.x

[13] Dong YF, Kataoka K, Tokutomi Y, et al. Perindopril, a centrally active angiotensin-converting enzyme inhibitor, prevents cognitive impairment in mouse models of Alzheimer's disease. *FASEB J*. 2011;25(9):2911–2920. doi:10.1096/fj.11-182873

[14] Labandeira-Garcia JL, Rodríguez-Perez AI, Garrido-Gil P, Rodriguez-Pallares J, Lanciego JL, Guerra MJ. Brain renin-angiotensin system and microglial polarization: implications for aging and neurodegeneration. *Front Aging Neurosci*. 2017;9:129. doi:10.3389/fnagi.2017.00129

[15] Sink KM, Leng X, Williamson J, et al. Angiotensin-converting enzyme inhibitors and cognitive decline in older adults with hypertension: results from the Cardiovascular Health Study. *Arch Intern Med*. 2009;169(13):1195–1202. doi:10.1001/archinternmed.2009.175

[16] Gao Y, O'Caoimh R, Healy L, et al. Effects of centrally acting ACE inhibitors on the rate of cognitive decline in dementia. *BMJ Open*. 2013;3(7):e002881. doi:10.1136/bmjopen-2013-002881

[17] Fazal K, Perera G, Khondoker M, Howard R, Stewart R. Associations of centrally acting ACE inhibitors with cognitive decline and survival in Alzheimer's disease. *BJPsych Open*. 2017;3(4):158–164. doi:10.1192/bjpo.bp.116.004184

[18] Yamada A, Sakurai T, Ochi D, Mitsuyama E, Yamauchi K, Abe F. Novel angiotensin I-converting enzyme inhibitory peptide derived from bovine casein. *Food Chem*. 2013;141(4):3781–3789. doi:10.1016/j.foodchem.2013.06.089

[19] Yamada A, Sakurai T, Ochi D, Mitsuyama E, Yamauchi K, Abe F. Antihypertensive effect of the bovine casein-derived peptide Met-Lys-Pro. *Food Chem*. 2015;172:441–446. doi:10.1016/j.foodchem.2014.09.098

[20] Min LJ, Kobayashi Y, Mogi M, et al. Administration of bovine casein-derived peptide prevents cognitive decline in Alzheimer disease model mice. *PLoS One*. 2017;12(2):e0171515. doi:10.1371/journal.pone.0171515

[21] Solfrizzi V, Panza F, Frisardi V, et al. Diet and Alzheimer's disease risk factors or prevention: the current evidence. *Expert Rev Neurother*. 2011;11(5):677–708. doi:10.1586/ern.11.56

[22] Mohs RC, Rosen WG, Davis KL. The Alzheimer's disease assessment scale: an instrument for assessing treatment efficacy. *Psychopharmacol Bull*. 1983;19(3):448–450.

[23] Homma A, Fukuzawa K, Tsukada Y, Ishii T, Hasegawa K, Mohs RC. Development of a Japanese version of the Alzheimer's disease assessment scale (ADAS) [in Japanese]. *Jpn J Geriatr Psychiatr*. 1992;3(6):647–655.

[24] Imai Y, Hasegawa K. The Revised Hasegawa's Dementia Scale (HDS-R)-Evaluation of its usefulness as a screening test for dementia. *J Hong Kong Coll Psychiatr*. 1994;4(SP2):20–24.

[25] Nasreddine ZS, Phillips NA, Bédirian V, et al. The Montreal Cognitive Assessment, MoCA: a brief screening tool for mild cognitive impairment. *J Am Geriatr Soc*. 2005;53(4):695–699. doi:10.1111/j.1532-5415.2005.53221.x

[26] Fujiwara Y, Suzuki H, Yasunaga M, et al. Brief screening tool for mild cognitive impairment in older Japanese: validation of the Japanese version of the Montreal Cognitive Assessment. *Geriatr Gerontol Int*. 2010;10(3):225–232. doi:10.1111/j.1447-0594.2010.00585.x

[27] Ware JE, Kosinski M, Dewey JE, Gandek B. How to score and interpret single-item health status measures: a manual for users of the SF-8 Health Survey. Lincoln, RI: QualityMetric, Inc., Boston.

[28] Yesavage JA, Brink TL, Rose TL, et al. Development and validation of a geriatric depression screening scale: a preliminary report. *J Psychiatr Res*. 1982;17(1):37–49. doi:10.1016/0022-3956(82)90033-4

[29] Sugishita K, Sugishita M, Hemmi I, Asada T, Tanigawa T. A Validity and Reliability Study of the Japanese Version of the Geriatric Depression Scale 15 (GDS-15-J). *Clin Gerontol*. 2017;40(4):233–240. doi:10.1080/07317115.2016.1199452

[30] Solfrizzi V, Capurso C, D'Introno A, et al. Lifestyle-related factors in predementia and dementia syndromes. *Expert Rev Neurother*. 2008;8(1):133–158. doi:10.1586/14737175.8.1.133

[31] Yurko-Mauro K, Alexander DD, Van Elswyk ME. Docosahexaenoic acid and adult memory: a systematic review and meta-analysis. *PLoS One*. 2015;10(3):e0120391. doi:10.1371/journal.pone.0120391

[32] Zhang Y, Chen J, Qiu J, Li Y, Wang J, Jiao J. Intakes of fish and polyunsaturated fatty acids and mild-to-severe cognitive impairment risks: a dose-response meta-analysis of 21 cohort studies. *Am J Clin Nutr*. 2016;103(2):330–340. doi:10.3945/ajcn.115.124081

[33] Hata J, Ohara T, Katakura Y, et al. Association between serum β-alanine and risk of dementia. *Am J Epidemiol*. 2019;188(9):1637–1645. doi:10.1093/aje/kwz116

[34] Guerrero-Berroa E, Luo X, Schmeidler J, et al. The MMSE orientation for time domain is a strong predictor of subsequent cognitive decline in the elderly. *Int J Geriatr Psychiatry*. 2009;24(12):1429–1437. doi:10.1002/gps.2282

[35] Shimbo A, Izawa EI, Fujisawa S. Scalable representation of time in the hippocampus. *Sci Adv*. 2021;7(6):eabd7013. doi:10.1126/sciadv.abd7013

[36] Giannakopoulos P, Gold G, Duc M, Michel JP, Hof PR, Bouras C. Neural substrates of spatial and temporal disorientation in Alzheimer's disease. *Acta Neuropathol*. 2000;100(2):189–195. doi:10.1007/s004019900166

[37] Tanaka M, Dohgu S, Komabayashi G, et al. Brain-transportable dipeptides across the blood–brain barrier in mice. *Sci Rep*. 2019;9(1):5769. doi:10.1038/s41598-019-42099-9

[38] Tanaka M, Kiyohara H, Yoshino A, et al. Brain-transportable soy dipeptide, Tyr-Pro, attenuates amyloid β peptide$_{25–35}$-induced memory impairment in mice. *NPJ Sci Food*. 2020;4:7. doi:10.1038/s41538-020-0067-3

[39] Kueper JK, Speechley M, Montero-Odasso M. The Alzheimer's Disease Assessment Scale-Cognitive Subscale (ADAS-Cog): modifications and responsiveness in pre-dementia populations. A narrative review. *J Alzheimers Dis*. 2018;63(2):423–444. doi:10.3233/JAD-170991

[40] Ueckert S, Plan EL, Ito K, et al. Improved utilization of ADAS-cog assessment data through item response theory based pharmacometric modeling. *Pharm Res*. 2014;31(8):2152–2165. doi:10.1007/s11095-014-1315-5

[41] Yuda N, Tanaka M, Yamada A, et al. Antihypertensive effect of the casein-derived peptide Met-Lys-Pro in individuals with high-normal blood pressure or grade 1 hypertension –A randomized, double-blind, placebo-controlled, parallel-group trial–. *Jpn Pharmacol Ther*. 2018;46(4):529–537.

Chapter 4

Safety Evaluation of High-Dose Intake of Casein-Derived Met-Lys-Pro in Healthy Adults: A Randomized Controlled Trial

4.1 Introduction

Cognitive decline is considered a normal consequence of aging; the total number of individuals with dementia was 50 million in 2015 and is predicted to reach 82 million by 2030 and 152 million by 2050 [1]. Therefore, prevention and care for cognitive decline are issues that need to be addressed worldwide. AD is the most common cause of dementia, which impairs memory, thinking skills, and behavior. The physiological changes leading to AD begin to develop decades before the earliest clinical symptoms appear, and when symptoms become clinically apparent, disease progression is too advanced for treatment [2]. Therefore, current research has focused on intervention in cognitively healthy individuals at risk of developing AD to reduce its incidence and prevalence [3].

Recently, centrally active ACE inhibitors have gained attention as a new therapy for AD [4]. ACE activity in the brains of patients with AD is elevated compared with that in the brains of non-dementia patients [5,6]. In addition, ACE generates elevated levels of Ang II in the brains of patients with AD [7], which promotes neurodegeneration and brain aging [8,9]. Therefore, ACE inhibition represents a potential neuroprotective approach for AD. Especially, centrally active ACE inhibitors with brain-penetrating ability that prevent the progression of neurodegeneration potentially protect against the development of AD [4,10–12].

Food-derived bioactive peptides have been considered for human health applications beyond their caloric value [13–17]. For example, these peptides have potential anti-hypertensive, anti-oxidant, lipid-lowering, immunomodulatory, anti-microbial, anti-cancer, anti-diabetic, and mineral binding activities. The anti-hypertensive effect of ACE inhibition is one of the most studied properties of food-derived bioactive peptides [16].

The bovine casein-derived tripeptide MKP was identified as having a strong ACE inhibitory activity (IC_{50} = 0.43 μM) and anti-hypertensive effect in SHRs [18,19]. In Chapter 2, daily oral intake of 100 μg of MKP for 12 weeks safely lowered SBP in humans with high-normal BP or grade 1 hypertension [20]. Furthermore, orally administered MKP significantly attenuated cognitive decline *in vivo* [21]. In stroke-prone SHRs, MKP administration showed neuroprotective effects by regulating cerebral circulation and corticoid secretion [22]. In Chapter 3, a clinical trial for community-dwelling adults without dementia demonstrated that daily oral intake of 200 μg of MKP over 24 weeks could improve orientation, with good tolerability and without treatment-related AEs [23]. Therefore, casein-derived MKP intake may be a safe preventive measure against not only hypertension but also cognitive decline.

Casein-derived MKP shows a high solubility in water and can be blended into a variety of foods. However, the ease of intake may lead to the possibility of overdose. Therefore, a 4-week, randomized, double-blind, placebo-controlled trial in healthy adults was conducted to evaluate the safety of daily intake of 1000 μg of MKP, which is five times the minimum effective dose for cognitive improvement [23].

4.2 Methods

4.2.1 Participants

Healthy adult volunteers aged ≥ 20 years living in Maebashi and the surrounding areas were recruited in January 2020. Exclusion criteria included: history of serious illness (e.g., heart failure, myocardial infarction, malignant tumor); treatment with medication for lifestyle-related disease (e.g., diabetes, hypertension, dyslipidemia); digestive tract diseases or history of

gastrointestinal surgery; serious allergies to medicine or food; history of drug dependence or alcoholism; participation or planned participation in other clinical studies; ineligibility owing to physician's diagnosis based on clinical laboratory test results and interview; pregnancy, lactation, or planning to get pregnant during the study period.

The study protocol was examined and approved by the Ethical Committee of Kobuna Orthopedics Clinic (approval code: MK1911-3). The study was conducted in accordance with the Declaration of Helsinki and the Ethical Guidelines for Medical and Health Research Involving Human Subjects (Ministry of Education, Culture, Sports, Science and Technology; Ministry of Health, Labor and Welfare, Japan). After receiving a detailed explanation of the objectives and procedures of the study, all participants provided written informed consent and were informed that they were free to withdraw at any time without obligation. This trial was registered at the UMIN-CTR as UMIN000038963.

4.2.2 Study Products

All study products were delivered as powdered beverages in sachets. The composition of the study products is detailed in Table 4-1. Morinaga Milk Industry (Tokyo, Japan) prepared the placebo along with the test product containing the equivalent of five times the minimum effective dose for cognitive improvement [23]. Specifically, one sachet of the test powder contained 1000 μg of MKP in 5 g of casein hydrolysate, and one sachet of the placebo powder contained 5 g of dextrin with no detectable MKP. The two different products were matched for appearance.

Table 4-1 Composition of the study products.

Components	MKP (1 sachet/day)	Placebo (1 sachet/day)
Energy (kcal)	25.0	25.0
Protein (g)	4.4	0.0
Fat (g)	0.0	0.0
Carbohydrates (g)	1.8	6.2
Sodium (mg)	51.0	106.0
Casein hydrolysate containing MKP (g/day)	5.0	0.0
MKP (μg/day)	1000.0	0.0
Dextrin (g/day)	0.0	5.0

4.2.3 Procedures

This study was designed as a randomized, double-blind, placebo-controlled trial, conducted at Maebashi North Hospital in Gunma, Japan between January and March 2020. Eligible participants were randomly allocated to receive either casein-derived MKP or placebo in a 1:1 ratio by a person not directly involved in the study using computer-generated lists of random numbers via the randomly permuted block method. Participants, physicians, researchers assessing outcomes, and researchers conducting statistical analyses were blinded to treatment group allocation over the study duration.

This study consisted of a 2-week pre-intake period, a 4-week intake period, and a 2-week follow-up period. During the intake period, participants were instructed to consume casein-derived MKP or placebo powder with 150–200 mL of water daily at their leisure. All participants were instructed to avoid marked alterations to diet or lifestyle and excessive drinking or eating throughout the study period. The participants were also asked to maintain

diary records of items related to the study products, illness, use of medications, and hospital visits. Treatment compliance was assessed by inspecting the diaries. Participants were instructed to visit the clinic for screening 3 weeks prior to commencing the experiment, at week 0, the beginning of the experimental period, at weeks 2 and 4 during the experiment, and at week 6 after a follow-up period. At these visits, participants were interviewed, anthropometric and BP measurements were made, and blood and urine samples were collected. Physicians interviewed participants throughout the study to assess the physical condition, subjective symptoms, and any AEs.

4.2.4 Anthropometric and Blood Pressure Measurements

Body height was measured only during screening. BW, SBP, DBP, and pulse rate were measured at all clinic visits. BW was measured using a weighing scale with 200 kg capacity, with ranges of 0–100 kg in 100 g resolution and 100–200 kg in 200 g resolution (AD-6207A, A&D Company, Tokyo, Japan). During BW measurement the participants wore light clothes and no shoes. BMI was calculated as BW (in kilograms) divided by the square of height (in meters). BP and pulse rate were measured using an automatic BP monitor (TM-2656VPW, A&D Company, Tokyo, Japan) in a seated position.

4.2.5 Blood and Urine Analyses

Blood and urine analyses were performed at all clinic visits. White blood cell count, red blood cell count, hemoglobin, hematocrit, platelet count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), MCH concentration, and leukocyte differentiation

(percentage of neutrophils, lymphocytes, monocytes, eosinophils, and basophils) were recorded. Aspartate aminotransferase, alanine aminotransferase, lactic dehydrogenase, total bilirubin, alkaline phosphatase, γ-glutamyl transpeptidase, creatine kinase (CK), fasting blood glucose, hemoglobin A1c (HbA1c), total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglyceride, total protein, albumin, blood urea nitrogen, creatinine, uric acid (UA), sodium, chloride, potassium, calcium, inorganic phosphorus, magnesium, and serum iron were also recorded. Urine specific gravity (USG), urine pH (U-pH), urine protein (U-pro), urine glucose (U-glu), urine urobilinogen (U-uro), urine bilirubin (U-bil), urine ketone body (U-ket), and occult blood reaction (OBR) were measured. Hematological examination, blood biochemical examination, and urinalysis were performed by the LSI Medience Corporation (Tokyo, Japan).

4.2.6 Safety Monitoring

All participants were monitored throughout the study for AEs and side-effects. Safety monitoring included a questionnaire on general health and the occurrence of any health-related events. Physicians considered the results of interviews and participants' diaries at weeks 0, 2, 4, and 6, and determined the relationships between any AEs and ingestion of study products while remaining blinded to group allocation.

4.2.7 Sample Size

As this was the first clinical trial to evaluate the safety of high-dose intake of casein-derived MKP, the sample size was determined by referring to a similar trial on food ingredient [24].

4.2.8 Statistical Analysis

Statistical analysis was based on the full dataset, defined as all randomized participants receiving study treatment with at least one test result after treatment. Values were presented as mean ± SE except for values at the screening period, which were expressed as mean ± SD. For continuous variables, statistically significant differences between the study groups were examined using unpaired *t*-test, and the changes from baseline values within the same groups were analyzed using paired *t*-test. For categorical data, statistically significant differences between the study groups were examined using Fisher's exact test. For urinalyses except for specific gravity and pH, data were coded as 0 or 1 as within or outside the reference range, respectively. The data expressed as a matrix of the number of participants and codes was then analyzed using Fisher's exact test. Findings were regarded as significant at $P < 0.05$ according to a two-tailed test. All statistical analyses were performed using IBM SPSS Statistics version 23 (IBM Corp., Armonk, NY, USA).

4.3 Results

4.3.1 Participants

Of a total of 90 candidates screened for the study, 30 participants (40.3 ± 12.1 years) were enrolled and randomly allocated into the MKP (n = 15) or placebo (n = 15) groups (Figure 4-1). All participants completed the study. Table 4-2 shows the characteristics of the participants at the screening period. The two groups did not differ significantly in demographic variables, including blood and urine analyses. The demographic means of both groups were within the reference range. The treatment compliance rate of both groups was 100%.

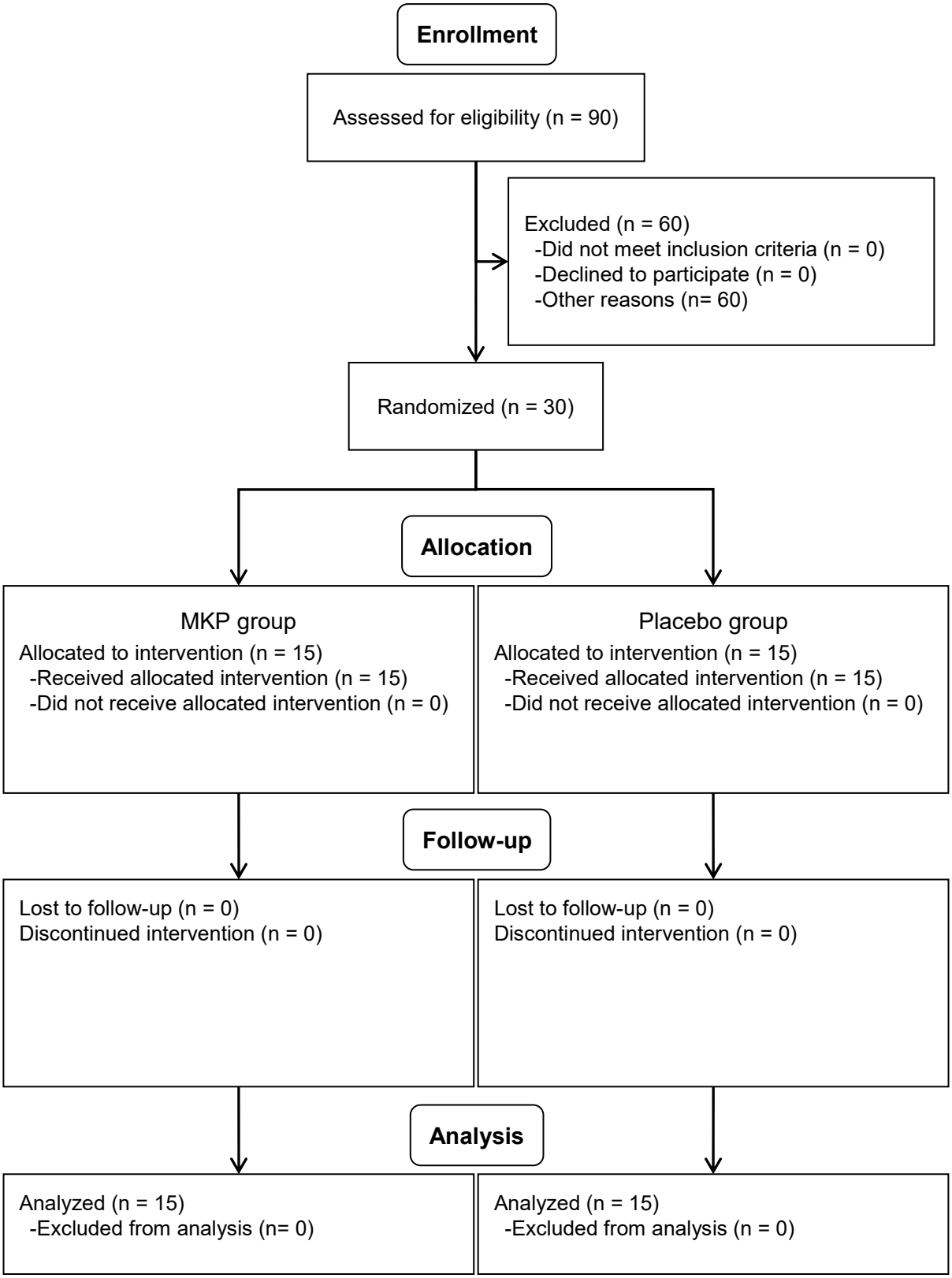

Figure 4-1 Study flow diagram.

Table 4-2 Participant characteristics at the screening period.

Characteristics	MKP (n = 15)	Placebo (n = 15)
Male/Female (n)	7/8	8/7
Age (years)	40.3 ± 12.8	40.3 ± 11.8
Body height (cm)	166.2 ± 4.2	163.8 ± 8.0
BW (kg)	61.8 ± 7.8	61.5 ± 10.7
BMI (kg/m^2)	22.3 ± 2.5	22.9 ± 2.8
SBP (mmHg)	121.8 ± 7.7	123.9 ± 8.0
DBP (mmHg)	72.8 ± 7.1	73.6 ± 9.0
Pulse rate (beats/min)	70.5 ± 8.2	68.8 ± 6.8

Data represent numbers of participants or mean ± SD. BW, body weight; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure.

4.3.2 Anthropometric and Blood Pressure Measurements

A summary of the anthropometric and BP measurements at baseline (week 0), during treatment (weeks 2 and 4), and post-treatment (week 6) is presented in Table 4-3. DBP was significantly lower in the MKP group than in the placebo group at week 4 (mean difference with 95% CI: −6.20 [−11.33, −1.07] mmHg, $P = 0.020$). Moreover, DBP in the MKP group was significantly decreased at week 4 compared with that at week 0 (mean difference with 95% CI: −4.33 [−7.10, −1.57] mmHg, $P = 0.005$). The pulse rate in the placebo group was significantly increased at week 2 compared with that at week 0 (mean difference with 95% CI: 1.60 [0.01, 3.19] beats/min, $P = 0.049$). BW, BMI, and SBP did not significantly vary between the groups or within each group. No clinical problems were noted in anthropometric or BP measurements.

Table 4-3 Summary of anthropometric and BP measurements.

Variable	Group	Week 0	Week 2	Week 4	Week 6
BW (kg)	MKP	61.8 ± 2.0	61.7 ± 2.0	61.7 ± 2.1	61.6 ± 2.1
	Placebo	61.5 ± 2.8	61.5 ± 2.8	61.7 ± 2.7	61.6 ± 2.7
BMI (kg/m^2)	MKP	22.3 ± 0.6	22.3 ± 0.6	22.3 ± 0.7	22.3 ± 0.7
	Placebo	22.9 ± 0.7	22.9 ± 0.7	22.9 ± 0.7	22.9 ± 0.7
SBP (mmHg)	MKP	121.8 ± 2.0	121.7 ± 1.6	119.1 ± 1.8	119.4 ± 2.1
	Placebo	123.9 ± 2.1	124.2 ± 1.9	121.3 ± 2.5	120.9 ± 2.6
DBP (mmHg)	MKP	72.8 ± 1.8	71.2 ± 2.1	68.5 ± 1.9**†	70.8 ± 2.4
	Placebo	73.6 ± 2.3	74.3 ± 2.0	74.7 ± 1.7	73.7 ± 1.8
Pulse rate (beats/min)	MKP	70.5 ± 2.1	69.8 ± 2.0	69.5 ± 1.7	70.1 ± 1.7
	Placebo	68.8 ± 1.8	70.4 ± 1.7*	70.0 ± 1.7	70.1 ± 1.6

Data represent mean ± SE. *$P < 0.05$ and **$P < 0.01$ indicate significant difference compared with week 0 values according to paired *t*-test; †$P < 0.05$ indicates significant difference compared with the placebo group according to unpaired *t*-test. BP, blood pressure; BW, body weight; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure.

4.3.3 Blood and Urine Analyses

No clinical problems were apparent in the blood and urine analyses. Tables 4-4 and 4-5 give summaries of the hematology test results. The hematocrit in the MKP group was significantly decreased at week 6 compared with that at week 0 (mean difference with 95% CI: −0.97 [−1.56, −0.39] %, $P = 0.003$). MCV in the MKP group was significantly decreased at weeks 2, 4, and 6 compared with that at week 0 (mean difference with 95% CI: −0.87 [−1.45, −0.28], −0.87 [−1.59, −0.15], −1.20 [−1.76, −0.64] fL, $P = 0.007$, $P = 0.022$, and $P < 0.001$, respectively). MCH in the MKP group was significantly decreased at week 2 compared with that at week 0 (mean difference with 95% CI: −0.19 [−0.385, −0.002] pg, $P = 0.048$). In the placebo group, the eosinophils/leukocytes ratio was significantly increased at week 6 compared with that at

week 0 (mean difference with 95% CI: 0.52 [0.02, 1.02] %, P = 0.042). The basophils/leukocytes ratio was significantly lower in the MKP group than in the placebo group at weeks 2, 4, and 6 (mean difference with 95% CI: −0.22 [−0.42, −0.01], −0.21 [−0.39, −0.02], −0.26 [−0.46, −0.06] %, P = 0.034, P = 0.032, and P = 0.012, respectively). Moreover, in the placebo group, the basophils/leukocytes ratio was significantly increased at week 6 compared with that at week 0 (mean difference with 95% CI: 0.20 [0.07, 0.33] %, P = 0.005). Other blood test variables did not significantly differ between the groups or within each group.

Tables 4-6, 4-7, and 4-8 show summaries of the blood biochemistry analysis. At week 0, the mean CK in the placebo group exceeded the reference range, because the value of one male in the placebo group was abnormal (2296 U/L). A re-examination was performed one week later, and as this yielded a normal value (154 U/L), the participant was allowed to continue the experiment. CK in the MKP group was significantly increased at week 2 compared with that at week 0 (mean difference with 95% CI: 23.73 [3.62, 43.9] U/L, P = 0.024). UA in the MKP group was significantly decreased at week 4 compared with that at week 0 (mean difference with 95% CI: −0.37 [−0.71, −0.04] mg/dL, P = 0.030). HbA1c in the placebo group was significantly decreased at week 6 compared with that at week 0 (mean difference with 95% CI: −0.06 [−0.11, −0.01] %, P = 0.023). At week 6, chloride was higher in the MKP group than in the placebo group (mean difference with 95% CI: 1.67 [0.07, 3.27] mEq/L, P = 0.042). Calcium in the placebo group was significantly decreased at week 6 compared with that at week 0 (mean difference with 95% CI: −0.16 [−0.28, −0.04] mg/dL, P = 0.011). Magnesium in the MKP group was significantly decreased at week 4 compared with that at week 0 (mean difference with 95% CI: −0.16 [−0.28, −0.04] mg/dL, P = 0.028). Other blood biochemistry variables did not

significantly differ between the groups or within each group.

Table 4-9 summarizes the USG and U-pH determinations. At week 6, the U-pH was significantly lower in the MKP group than in the placebo group (mean difference with 95% CI: −0.57 [−1.07, −0.06], $P = 0.029$). Moreover, the U-pH in the placebo group was significantly increased at week 6 compared with that at week 0 (mean difference with 95% CI: 0.57 [0.09, 1.04], $P = 0.023$). The U-pH did not significantly differ at any other time point, and the USG test results did not significantly vary at all between the groups or within each group. Similarly, U-pro, U-glu, U-uro, U-bil, U-ket, and OBR did not significantly vary between the groups (Table 4-10).

Table 4-4 Summary of hematological examination (blood cell count).

Variable	Reference range	Group	Week 0	Week 2	Week 4	Week 6
WBC (× 10^2/μL)	33–90	MKP	56.5 ± 3.4	57.3 ± 4.1	59.5 ± 4.2	58.1 ± 3.9
		Placebo	57.3 ± 4.5	57.0 ± 4.4	57.1 ± 3.8	55.9 ± 3.8
RBC (× 10^4/μL)	M: 430–570	MKP	473.1 ± 9.4	471.0 ± 9.5	474.9 ± 9.4	468.1 ± 9.5
	F: 380–500	Placebo	478.1 ± 11.7	483.3 ± 11.3	475.9 ± 11.8	476.5 ± 12.4
Hb (g/dL)	M: 13.5–17.5	MKP	14.4 ± 0.4	14.2 ± 0.4	14.4 ± 0.4	14.1 ± 0.4
	F: 11.5–15.0	Placebo	14.5 ± 0.4	14.6 ± 0.4	14.4 ± 0.4	14.5 ± 0.4
Ht (%)	M: 39.7–52.4	MKP	45.1 ± 1.0	44.5 ± 1.0	44.8 ± 1.0	44.1 ± 1.0**
	F: 34.8–45.0	Placebo	45.3 ± 1.1	45.7 ± 1.0	44.9 ± 1.0	44.9 ± 1.0
PLT (× 10^4/μL)	14.0–34.0	MKP	27.8 ± 1.3	27.9 ± 1.2	27.7 ± 1.3	28.0 ± 1.4
		Placebo	28.0 ± 1.5	28.4 ± 1.6	28.3 ± 1.7	28.0 ± 1.5
MCV (fL)	85.0–102.0	MKP	95.3 ± 1.1	94.4 ± 1.1**	94.4 ± 1.1*	94.1 ± 0.9**
		Placebo	94.7 ± 1.2	94.7 ± 1.0	94.4 ± 1.0	94.4 ± 1.0
MCH (pg)	28.0–34.0	MKP	30.3 ± 0.4	30.1 ± 0.4*	30.2 ± 0.4	30.1 ± 0.4
		Placebo	30.4 ± 0.4	30.2 ± 0.4	30.2 ± 0.4	30.4 ± 0.4
MCHC (%)	30.2–35.1	MKP	31.9 ± 0.2	31.9 ± 0.2	32.0 ± 0.2	32.0 ± 0.2
		Placebo	32.1 ± 0.3	31.9 ± 0.2	32.0 ± 0.2	32.2 ± 0.2

Data represent mean ± SE. *$P < 0.05$ and **$P < 0.01$ indicate significant difference compared with week 0 values according to paired *t*-test. WBC, white blood cell count; RBC, red blood cell count; Hb, hemoglobin; Ht, hematocrit; PLT, platelet count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; M, male; F, female.

Table 4-5 Summary of hematological examination (differential leukocyte ratio).

Variable	Reference range	Group	Week 0	Week 2	Week 4	Week 6
Neut (%)	40.0–75.0	MKP	54.0 ± 2.5	56.7 ± 1.3	56.4 ± 1.9	56.7 ± 2.0
		Placebo	54.2 ± 2.8	55.0 ± 2.2	53.6 ± 1.2	52.5 ± 1.8
Lympho (%)	18.0–49.0	MKP	36.7 ± 2.5	34.6 ± 1.5	34.4 ± 2.0	34.4 ± 1.9
		Placebo	36.7 ± 2.8	36.0 ± 2.0	37.4 ± 1.3	37.8 ± 1.8
Mono (%)	2.0–10.0	MKP	5.8 ± 0.3	5.7 ± 0.5	5.8 ± 0.3	5.6 ± 0.3
		Placebo	5.8 ± 0.3	6.0 ± 0.4	5.5 ± 0.4	5.7 ± 0.3
Eosino (%)	0.0–8.0	MKP	3.0 ± 0.5	2.5 ± 0.3	2.9 ± 0.6	2.8 ± 0.5
		Placebo	2.7 ± 0.2	2.3 ± 0.2	2.9 ± 0.2	3.2 ± 0.3*
Baso (%)	0.0–2.0	MKP	0.5 ± 0.1	0.4 ± 0.1†	0.5 ± 0.1†	0.5 ± 0.1†
		Placebo	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.8 ± 0.1**

Data represent mean ± SE. *$P < 0.05$ and **$P < 0.01$ indicate significant difference compared with week 0 values according to paired *t*-test; †$P < 0.05$ indicates a significant difference compared with the placebo group according to unpaired *t*-test. Neut, neutrophils/leukocytes; Lympho, lymphocytes/leukocytes; Mono, monocytes/leukocytes; Eosino, eosinophils/leukocytes; Baso, basophils/leukocytes; M, male; F, female.

Table 4-6 Summary of blood biochemistry (proteins, pigment, and enzymes).

Variable	Reference range	Group	Week 0	Week 2	Week 4	Week 6
TP (g/dL)	6.7–8.3	MKP	7.2 ± 0.1	7.2 ± 0.1	7.1 ± 0.1	7.1 ± 0.1
		Placebo	7.2 ± 0.1	7.3 ± 0.1	7.1 ± 0.1	7.1 ± 0.1
ALB (g/dL)	3.8–5.2	MKP	4.4 ± 0.1	4.4 ± 0.1	4.4 ± 0.1	4.4 ± 0.1
		Placebo	4.5 ± 0.1	4.5 ± 0.1	4.4 ± 0.1	4.5 ± 0.1
TB (mg/dL)	0.2–1.2	MKP	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
		Placebo	0.8 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	1.0 ± 0.1
AST (U/L)	10–40	MKP	19.7 ± 1.4	20.6 ± 1.0	19.0 ± 1.5	19.4 ± 1.4
		Placebo	20.3 ± 3.0	18.7 ± 1.3	21.5 ± 3.2	18.5 ± 1.7
ALT (U/L)	5–45	MKP	14.0 ± 1.5	15.4 ± 1.4	14.3 ± 1.7	14.7 ± 1.7
		Placebo	15.9 ± 2.1	16.5 ± 2.6	20.5 ± 5.0	20.9 ± 5.7
LD (U/L)	120–240	MKP	173.1 ± 7.3	172.7 ± 7.5	168.5 ± 7.3	170.1 ± 7.0
		Placebo	178.5 ± 4.8	173.5 ± 6.0	176.1 ± 6.4	172.7 ± 4.2
ALP (U/L)	100–325	MKP	196.9 ± 13.9	191.9 ± 13.2	192.3 ± 12.6	193.1 ± 12.8
		Placebo	167.9 ± 8.8	167.0 ± 8.5	166.3 ± 10.3	167.8 ± 9.1
γ-GT (U/L)	M: ≤ 80	MKP	22.7 ± 5.3	22.0 ± 4.6	22.2 ± 5.3	22.9 ± 4.9
	F: ≤ 30	Placebo	21.0 ± 2.4	21.4 ± 2.9	22.5 ± 2.5	22.3 ± 2.5
CK (U/L)	M: 60–270	MKP	89.5 ± 11.8	113.3 ± 20.0*	97.6 ± 17.8	110.7 ± 22.6
	F: 40–150	Placebo	273.4 ± 147.0	103.0 ± 6.7	173.1 ± 69.6	94.8 ± 5.0

Data represent mean ± SE. *$P < 0.05$ indicates a significant difference compared with week 0 values according to paired *t*-test. TP, total protein; ALB, albumin; TB, total bilirubin; AST, aspartate transaminase; ALT, alanine aminotransferase; LD, lactate dehydrogenase; ALP, alkaline phosphatase; γ-GT, γ-glutamyltransferase; CK, creatine kinase; M, male; F, female.

Table 4-7 Summary of blood biochemistry (low molecular nitrogen compounds, carbohydrates, and lipids).

Variable	Reference range	Group	Week 0	Week 2	Week 4	Week 6
BUN (mg/dL)	8.0–20.0	MKP	12.9 ± 1.2	13.6 ± 1.1	13.4 ± 0.9	12.8 ± 1.0
		Placebo	11.8 ± 0.5	11.5 ± 0.5	11.4 ± 0.7	11.5 ± 0.5
CRE (mg/dL)	M: 0.61–1.04	MKP	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0
	F: 0.47–0.79	Placebo	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0
UA (mg/dL)	M: 3.8–7.0	MKP	5.1 ± 0.4	4.8 ± 0.3	4.7 ± 0.3*	5.0 ± 0.3
	F: 2.5–7.0	Placebo	5.1 ± 0.3	4.9 ± 0.3	5.0 ± 0.3	5.0 ± 0.3
FBG (mg/dL)	70–109	MKP	82.1 ± 2.2	82.7 ± 1.6	84.4 ± 2.2	80.6 ± 1.5
		Placebo	83.3 ± 2.3	83.3 ± 1.9	82.9 ± 1.8	80.9 ± 2.4
HbA1c (%)	4.6–6.2	MKP	5.3 ± 0.1	5.3 ± 0.1	5.3 ± 0.1	5.3 ± 0.1
		Placebo	5.4 ± 0.1	5.4 ± 0.1	5.3 ± 0.1	5.3 ± 0.1*
TC (mg/dL)	120–219	MKP	193.5 ± 7.9	194.4 ± 7.6	191.0 ± 8.8	190.9 ± 7.2
		Placebo	197.1 ± 5.4	203.7 ± 6.6	193.7 ± 6.0	200.9 ± 5.8
LDL-C (mg/dL)	65–139	MKP	110.0 ± 6.7	110.4 ± 7.1	107.7 ± 7.7	109.1 ± 6.6
		Placebo	109.9 ± 5.2	117.1 ± 6.6	108.4 ± 5.8	116.2 ± 6.1
HDL-C (mg/dL)	M: 40–85	MKP	65.7 ± 2.5	67.7 ± 2.7	66.8 ± 2.5	67.2 ± 2.3
	F: 40–95	Placebo	68.5 ± 3.7	71.5 ± 3.9	67.7 ± 3.4	71.1 ± 3.6
TG (mg/dL)	30–149	MKP	87.0 ± 10.1	80.7 ± 12.7	80.7 ± 8.5	69.8 ± 5.3
		Placebo	87.6 ± 9.1	73.7 ± 7.5	85.7 ± 8.0	73.1 ± 7.7

Data represent mean ± SE. *$P < 0.05$ indicates a significant difference compared with week 0 values according to paired *t*-test. BUN, blood urea nitrogen; CRE, creatinine; UA, uric acid; FBG, fasting blood glucose; HbA1c, hemoglobin A1c; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride; M, male; F, female.

Table 4-8 Summary of blood biochemistry (electrolytes and trace element).

Variable	Reference range	Group	Week 0	Week 2	Week 4	Week 6
Na (mEq/L)	137–147	MKP	140.8 ± 0.5	140.5 ± 0.4	140.1 ± 0.3	140.2 ± 0.4
		Placebo	140.5 ± 0.4	139.8 ± 0.6	140.3 ± 0.5	139.5 ± 0.5
Cl (mEq/L)	98–108	MKP	105.1 ± 0.6	104.3 ± 0.4	104.7 ± 0.4	105.5 ± 0.5†
		Placebo	104.1 ± 0.5	103.1 ± 0.5	104.3 ± 0.6	103.8 ± 0.6
K (mEq/L)	3.5–5.0	MKP	4.5 ± 0.1	4.3 ± 0.1	4.4 ± 0.1	4.4 ± 0.1
		Placebo	4.3 ± 0.1	4.4 ± 0.1	4.3 ± 0.1	4.3 ± 0.1
Ca (mg/dL)	8.4–10.4	MKP	9.6 ± 0.1	9.5 ± 0.1	9.5 ± 0.1	9.5 ± 0.1
		Placebo	9.6 ± 0.1	9.6 ± 0.1	9.4 ± 0.1	9.4 ± 0.1*
IP (mg/dL)	2.5–4.5	MKP	3.6 ± 0.1	3.5 ± 0.1	3.5 ± 0.2	3.7 ± 0.1
		Placebo	3.9 ± 0.2	3.8 ± 0.1	3.8 ± 0.1	3.7 ± 0.1
Mg (mg/dL)	1.9–2.5	MKP	2.2 ± 0.0	2.1 ± 0.0	2.1 ± 0.0*	2.1 ± 0.0
		Placebo	2.1 ± 0.0	2.2 ± 0.0	2.1 ± 0.0	2.1 ± 0.0
Fe (μg/dL)	M: 50–200	MKP	106.8 ± 15.2	107.8 ± 9.3	122.9 ± 13.0	118.1 ± 6.7
	F: 40–180	Placebo	109.9 ± 12.5	139.4 ± 18.3	103.2 ± 12.0	119.7 ± 14.5

Data represent mean ± SE. *$P < 0.05$ indicates significant difference compared with week 0 values according to paired *t*-test; †$P < 0.05$ indicates a significant difference compared with the placebo group according to unpaired *t*-test. Na, sodium; Cl, chloride; K, potassium; Ca, calcium; IP, inorganic phosphorus; Mg, magnesium; Fe, serum iron; M, male; F, female.

Table 4-9 Summary of USG and U-pH.

Variable	Reference range	Group	Week 0	Week 2	Week 4	Week 6
USG	1.006–1.030	MKP	1.018 ± 0.002	1.018 ± 0.002	1.017 ± 0.002	1.014 ± 0.002
		Placebo	1.015 ± 0.002	1.015 ± 0.002	1.015 ± 0.002	1.016 ± 0.002
U-pH	5.0–7.5	MKP	6.4 ± 0.2	6.7 ± 0.2	6.5 ± 0.2	6.1 ± 0.2†
		Placebo	6.1 ± 0.1	6.3 ± 0.2	6.3 ± 0.2	6.6 ± 0.2*

Data indicate mean ± SD. *$P < 0.05$ indicates a significant difference compared with the week 0 values according to paired *t*-test; †$P < 0.05$ indicates a significant difference compared with the placebo group according to unpaired *t*-test. USG, urine specific gravity; U-pH, urine pH.

Table 4-10 Summary of urinalysis results.

Variable	Week	MKP (n = 15)		Placebo (n = 15)		*P* value
		Reference range		Reference range		
		Within	Outside	Within	Outside	
U-pro	0	15	0	15	0	NA
	2	14	1	15	0	1.00
	4	14	1	15	0	1.00
	6	15	0	15	0	NA
U-glu	0	15	0	15	0	NA
	2	15	0	15	0	NA
	4	15	0	15	0	NA
	6	15	0	15	0	NA
U-uro	0	15	0	15	0	NA
	2	15	0	15	0	NA
	4	15	0	15	0	NA
	6	15	0	15	0	NA
U-bil	0	15	0	15	0	NA
	2	15	0	15	0	NA
	4	15	0	15	0	NA
	6	15	0	15	0	NA
U-ket	0	15	0	15	0	NA
	2	15	0	15	0	NA
	4	15	0	15	0	NA
	6	15	0	15	0	NA
OBR	0	15	0	15	0	NA
	2	14	1	14	1	1.00
	4	14	1	14	1	1.00
	6	13	2	15	0	0.48

Data represent numbers of participants. *P* values were calculated using Fisher's exact test. U-pro, urine protein; U-glu, urine glucose; U-uro, urine urobilinogen; U-bil, urine bilirubin; U-ket, urine ketone body; OBR, occult blood reaction; NA, not applicable.

4.3.4 Safety

Nine AEs were reported by seven participants throughout the study, with one reported by one participant in the MKP group and eight reported by six participants in the placebo group. These included elevated serum iron (two participants in the placebo group), muscle pain (two participants in the placebo group), elevated CK (one participant in the MKP group and three participants in the placebo group), and elevated total bilirubin (one participant in the placebo group). All reported AEs were mild and judged to be unrelated to the intake of study products.

4.4 Discussion

Several dietary factors have preventive effects on cognitive decline and AD [25–28], and the bioactive peptides were demonstrated to exert preventive effects on cognitive decline in animal models and human studies [29]. In Chapter 3, casein-derived MKP has the potential to improve orientation in community-dwelling adults without dementia, with good tolerability, and no treatment-related AEs during 24 weeks of treatment and for 2 weeks after treatment [23]. A randomized, double-blind, placebo-controlled trial was conducted to examine the safety of a high-dose intake of casein-derived MKP in healthy adults and found that supplementation of 1000 μg of MKP for 4 weeks yielded no adverse outcomes. The trial showed significant differences in some parameters, but these changes did not appear to be clinically significant. Therefore, they were not considered to be clinical safety concerns attributable to the intake of the study products.

Nine AEs were reported by seven participants throughout the study. Elevated CK was the most common, occurring in one participant in the MKP group and three participants in the

placebo group. High levels of CK in healthy participants may be correlated with muscle damage following physical exercise [30], and all participants with elevated CK had been playing sports or doing physical work immediately before testing. Therefore, the CK elevation was unlikely to be caused by the consumption of the study products. Elevated serum iron, muscle pain, and elevated total bilirubin were also reported as AEs. However, all these AEs resolved without treatment and were therefore judged to be transient and unrelated to this study. These findings suggest that there are no clinical safety concerns associated with the high-dose intake of casein-derived MKP.

In Chapter 2, it was found that the daily ingestion of casein-derived MKP for 12 weeks was safe and effective in lowering SBP in people with high-normal BP or grade 1 hypertension [20]. In this trial, there was no significant difference in SBP between the MKP and placebo groups. This may be because the previous trial targeted people with high-normal BP, defined as SBP 130–139 mmHg and/or DBP 85–89 mmHg, or grade 1 hypertension, defined as SBP 140–159 mmHg and/or DBP 90–99 mmHg. In this trial, participants' mean BP before the intervention was not high (Table 4-2), although some participants with high-normal BP were included. The lack of effect of normotensives on BP was similarly reported in casein hydrolysate containing VPP and IPP with an ACE inhibitory effect [31]. On the other hand, DBP in the MKP group was significantly lower at week 4 than that in the placebo group. The reason may be the difference in the mean age of participants. DBP, unlike SBP, becomes less responsive with age. In this trial, the mean age of the participants was approximately 40 years old (Table 4-2), while the mean age in the previous trial approximately 50 years old (Table 2-2). Therefore, the mean age may have affected SBP. However, there were no cases of

hypotension, defined as SBP ≤ 90 mmHg and/or DBP ≤ 60 mmHg in this trial. Although further studies may be needed to evaluate the effect of high-dose casein-derived MKP on individuals with high BP, these results suggest that the high-dose intake of casein-derived MKP has little effect on normal BP.

The present study design was a well-controlled trial to evaluate the safety of a high-dose intake of casein-derived MKP. However, several limitations were noted. Firstly, the intervention was limited to 4 weeks. Secondly, it is still unknown whether the high-dose intake of casein-derived MKP affects the safety of individuals with the disease. It may be necessary to conduct an intervention with a longer test period to evaluate the safety of high-dose intake of casein-derived MKP on adults with and without the disease.

4.5 Conclusions

This randomized, double-blind, placebo-controlled trial assessed the safety of daily intake of 1000 μg of MKP for 4 weeks to healthy adult participants. No AEs that could be associated with the study products were observed in anthropometric and BP measurements, blood and urine analyses, or medical interviews. These findings suggest that casein-derived MKP may be safe for daily intake. Therefore, in the future, it may be applied as a safe preventive measure against hypertension and cognitive decline.

References

[1] World Health Organization. Risk reduction of cognitive decline and dementia. https://www.who.int/mental_health/neurology/dementia/guidelines_risk_reduction/en/. Published 2019. Accessed February 21, 2021.

[2] Jack CR Jr, Knopman DS, Jagust WJ, et al. Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. *Lancet Neurol*. 2010;9(1):119–128. doi:10.1016/S1474-4422(09)70299-6

[3] Crous-Bou M, Minguillón C, Gramunt N, Molinuevo JL. Alzheimer's disease prevention: from risk factors to early intervention. *Alzheimers Res Ther*. 2017;9(1):71. doi:10.1186/s13195-017-0297-z

[4] O'Caoimh R, Healy L, Gao Y, et al. Effects of centrally acting angiotensin converting enzyme inhibitors on functional decline in patients with Alzheimer's disease. *J Alzheimers Dis*. 2014;40(3):595–603. doi:10.3233/JAD-131694

[5] Arregui A, Perry EK, Rossor M, Tomlinson BE. Angiotensin converting enzyme in Alzheimer's disease increased activity in caudate nucleus and cortical areas. *J Neurochem*. 1982;38(5):1490–1492. doi:10.1111/j.1471-4159.1982.tb07930.x

[6] Miners JS, Ashby E, Van Helmond Z, et al. Angiotensin-converting enzyme (ACE) levels and activity in Alzheimer's disease, and relationship of perivascular ACE-1 to cerebral amyloid angiopathy. *Neuropathol Appl Neurobiol*. 2008;34(2):181–193. doi:10.1111/j.1365-2990.2007.00885.x

[7] Savaskan E, Hock C, Olivieri G, et al. Cortical alterations of angiotensin converting enzyme, angiotensin II and AT1 receptor in Alzheimer's dementia. *Neurobiol Aging*. 2001;22(4):541–546. doi:10.1016/s0197-4580(00)00259-1

[8] Dong YF, Kataoka K, Tokutomi Y, et al. Perindopril, a centrally active angiotensin-converting enzyme inhibitor, prevents cognitive impairment in mouse models of Alzheimer's disease. *FASEB J*. 2011;25(9):2911–2920. doi:10.1096/fj.11-182873

[9] Labandeira-Garcia JL, Rodríguez-Perez AI, Garrido-Gil P, Rodriguez-Pallares J, Lanciego JL, Guerra MJ. Brain renin-angiotensin system and microglial polarization: implications for aging and neurodegeneration. *Front Aging Neurosci*. 2017;9:129. doi:10.3389/fnagi.2017.00129

[10] Sink KM, Leng X, Williamson J, et al. Angiotensin-converting enzyme inhibitors and cognitive decline in older adults with hypertension: results from the Cardiovascular Health Study. *Arch Intern Med*. 2009;169(13):1195–1202. doi:10.1001/archinternmed.2009.175

[11] Gao Y, O'Caoimh R, Healy L, et al. Effects of centrally acting ACE inhibitors on the rate of cognitive decline in dementia. *BMJ Open*. 2013;3(7):e002881. doi:10.1136/bmjopen-2013-002881

[12] Fazal K, Perera G, Khondoker M, Howard R, Stewart R. Associations of centrally acting ACE inhibitors with cognitive decline and survival in Alzheimer's disease. *BJPsych Open*. 2017;3(4):158–164. doi:10.1192/bjpo.bp.116.004184

[13] Martínez-Maqueda D, Miralles B, Recio I, Hernández-Ledesma B. Antihypertensive peptides from food proteins: a review. *Food Funct*. 2012;3(4):350–361. doi:10.1039/c2fo10192k

[14] Cicero AFG, Fogacci F, Colletti A. Potential role of bioactive peptides in prevention and treatment of chronic diseases: a narrative review. *Br J Pharmacol*. 2017;174(11):1378–1394. doi:10.1111/bph.13608

[15] Mills S, Ross RP, Hill C, Fitzgerald GF, Stanton C. Milk intelligence: mining milk for bioactive substances associated with human health. *Int Dairy J*. 2011;21:377–401. doi:10.1016/j.idairyj.2010.12.011

[16] Udenigwe CC, Aluko RE. Food protein-derived bioactive peptides: production, processing, and potential health benefits. *J Food Sci*. 2012;77(1):R11–R24. doi:10.1111/j.1750-3841.2011.02455.x

[17] Manzanares P, Gandía M, Garrigues S, Marcos JF. Improving health-promoting effects of food-derived bioactive peptides through rational design and oral delivery strategies. *Nutrients*. 2019;11(10):2545. doi:10.3390/nu11102545

[18] Yamada A, Sakurai T, Ochi D, Mitsuyama E, Yamauchi K, Abe F. Novel angiotensin I-converting enzyme inhibitory peptide derived from bovine casein. *Food Chem*. 2013;141(4):3781–3789. doi:10.1016/j.foodchem.2013.06.089

[19] Yamada A, Sakurai T, Ochi D, Mitsuyama E, Yamauchi K, Abe F. Antihypertensive effect of the bovine casein-derived peptide Met-Lys-Pro. *Food Chem*. 2015;172:441–446. doi:10.1016/j.foodchem.2014.09.098

[20] Yuda N, Tanaka M, Yamada A, et al. Antihypertensive effect of the casein-derived peptide Met-Lys-Pro in individuals with high-normal blood pressure or grade 1 hypertension –A randomized, double-blind, placebo-controlled, parallel-group trial–. *Jpn Pharmacol Ther*. 2018;46(4):529–537.

[21] Min LJ, Kobayashi Y, Mogi M, et al. Administration of bovine casein-derived peptide prevents cognitive decline in Alzheimer disease model mice. *PLoS One*. 2017;12(2):e0171515. doi:10.1371/journal.pone.0171515

[22] Tada AM, Hamezah HS, Yanagisawa D, Morikawa S, Tooyama I. Neuroprotective effects of casein-derived peptide Met-Lys-Pro (MKP) in a hypertensive model. *Front Neurosci*. 2020;14:845. doi:10.3389/fnins.2020.00845

[23] Yuda N, Tanaka M, Yamauchi K, et al. Effect of the casein-derived peptide Met-Lys-Pro on cognitive function in community-dwelling adults without dementia: a randomized, double-blind, placebo-controlled trial. *Clin Interv Aging*. 2020;15:743–754. doi:10.2147/CIA.S253116

[24] Akazome Y, Kametani N, Kanda T, Shimasaki H, Kobayashi S. Evaluation of safety of excessive intake and efficacy of long-term intake of beverages containing apple polyphenols. *J Oleo Sci*. 2010;59(6):321–338. doi:10.5650/jos.59.321

[25] Solfrizzi V, Panza F, Frisardi V, et al. Diet and Alzheimer's disease risk factors or prevention: the current evidence. *Expert Rev Neurother*. 2011;11(5):677–708. doi:10.1586/ern.11.56

[26] Wu L, Sun D. Meta-analysis of milk consumption and the risk of cognitive disorders. *Nutrients*. 2016;8(12):824. doi:10.3390/nu8120824

[27] Yurko-Mauro K, Alexander DD, Van Elswyk ME. Docosahexaenoic acid and adult memory: a systematic review and meta-analysis. *PLoS One*. 2015;10(3):e0120391. doi:10.1371/journal.pone.0120391

[28] Zhang Y, Chen J, Qiu J, Li Y, Wang J, Jiao J. Intakes of fish and polyunsaturated fatty acids and mild-to-severe cognitive impairment risks: a dose-response meta-analysis of 21 cohort studies. *Am J Clin Nutr*. 2016;103(2):330–340. doi:10.3945/ajcn.115.124081

[29] Katayama S, Nakamura S. Emerging roles of bioactive peptides on brain health promotion. *Int J Food Sci Technol*. 2019;54:1949–1955. doi:10.1111/ijfs.14076

[30] Brancaccio P, Maffulli N, Buonauro R, Limongelli FM. Serum enzyme monitoring in sports medicine. *Clin Sports Med*. 2008;27(1):1–18. doi:10.1016/j.csm.2007.09.005

[31] Ishida Y, Shibata Y, Fukuhara I, Yano Y, Takehara I, Kaneko K. Effect of an excess intake of casein hydrolysate containing Val-Pro-Pro and Ile-Pro-Pro in subjects with normal blood pressure, high-normal blood pressure, or mild hypertension. *Biosci Biotechnol Biochem*. 2011;75(3):427–433. doi:10.1271/bbb.100560

Chapter 5

Concluding Remarks

In an aging society, extending healthy life expectancy has become a global challenge. Hypertension is a major risk factor for CVD, the most common cause of death in the world. ACE inhibition is one of the effective mechanisms for the prevention and treatment of hypertension. In recent years, it has been suggested that ACE inhibition not only lowers BP but also contributes towards the prevention and treatment of dementia. In particular, the results of several epidemiological studies have proposed that centrally active ACE inhibitors may improve cognitive function.

MKP was identified from the casein hydrolysate as the peptide with the highest contribution to the ACE inhibitory effect (IC_{50} = 0.43 μM). Casein-derived MKP attenuates BP elevation in SHRs and cognitive decline in the AD mice model. It was also suggested that orally administered MKP could be rapidly transferred intact into the bloodstream and MKP or its metabolite may reach the brain. On the other hand, the effect of casein-derived MKP intake on humans is not well known. This has been an issue limiting the utilization of casein-derived MKP in functional foods. The present study aimed to elucidate the effects of casein-derived MKP intake on BP and cognitive function in humans. It was found that the intake of casein-derived MKP lowered SBP and improved orientation in RCTs. Furthermore, this study demonstrated the safety of casein-derived MKP in humans.

In Chapter 2, the impact of casein-derived MKP intake on BP in individuals with high-normal BP or grade 1 hypertension was examined in a 12-week RCT. A total of 120 participants were randomly allocated to the MKP group (n = 60) or the placebo group (n = 60). The MKP group received two capsules daily, each containing 50 μg of MKP, while the placebo group received two capsules containing no detectable MKP for 12 weeks. A total of 103 participants

completed the trial. The reduction in SBP from baseline at week 12 was greater in the MKP group than in the placebo group (P = 0.0173, mean change −3.9 mmHg). No serious AEs due to casein-derived MKP ingestion were observed. It was known that orally ingested ^{14}C-MKP was transferred intact into the bloodstream in SHRs. Furthermore, Ang II-dependent vasoconstriction of thoracic aorta of rats was inhibited by MKP pre-treatment. These results suggest that also in humans, orally ingested MKP may be transferred intact into the bloodstream and lower SBP primarily via blockade of the RAS. It was reported that a decline in SBP led to lower mortality rate of stroke and coronary heart disease. Therefore, it is suggested that casein-derived MKP intake may contribute to the reduction of CVD risk.

In Chapter 3, the effect of casein-derived MKP intake on cognitive function in community-dwelling adults without dementia was investigated in a 24-week RCT. A total of 268 participants were randomly allocated to the MKP group (n = 134) or the placebo group (n = 134). The MKP group received four tablets daily, each containing 50 μg of MKP, while the placebo group received four tablets containing no detectable MKP for 24 weeks. Scores on the ADAS-cog were used as the primary outcome to compare cognitive function between the MKP and placebo groups. The results showed that orientation score, as measured by the respective ADAS-cog subscale, in the MKP group was significantly improved compared to the placebo group at week 24 (P = 0.022, Cohen's d = 0.30). No serious AEs due to casein-derived MKP intake were observed. Orientation is the ability to correctly identify place, time, and person and serves as a useful indicator of cognitive decline. Therefore, systematic dietary supplementation with casein-derived MKP may be a promising intervention for maintaining good cognitive function. Hippocampus plays central roles in spatial and temporal processing. In addition, it

was suggested that disorientation in humans may be associated with impairment in the hippocampus. The administration of casein-derived MKP suppressed inflammation and oxidative stress in the hippocampus in AD model mice. These results suggest that casein-derived MKP may improve orientation in non-demented individuals by maintaining normal hippocampal function.

In Chapter 4, the safety of high-dose intake of casein-derived MKP in healthy adults was evaluated by conducting a 4-week RCT. A total of 30 participants were randomly allocated to the MKP group (n = 15) or the placebo group (n = 15). The MKP group received test powder containing a daily dose of 1000 μg of MKP, five times the minimum effective dose for cognitive improvement, while the placebo group received dextrin powder containing no detectable MKP for 4 weeks. No clinical problems were observed in anthropometric measurements, BP, blood parameters, and urine parameters. No AEs owing to casein-derived MKP intake were observed. These findings suggest that intake of casein-derived MKP is recognized as safe.

RCT is a gold standard for proving the efficacy and safety in humans. The major strengths of the present study were that three RCTs were well-controlled trial design to evaluate the efficacy and safety of casein-derived MKP. On the other hand, there were some limitations of this study. First, the participants of this study were limited to a specific population. The effects of casein-derived MKP on individuals with severe hypertension and dementia were not clarified. Also, in healthy individuals, the effects of ethnicity, genetic background, and lifestyle on the efficacy of casein-derived MKP were not revealed. Second, the duration of intake was limited. The effects of a longer intake period on the efficacy of casein-derived MKP were not clear. Third, the evaluating methods were limited. For BP, 24-hour ambulatory BP, as well as office

BP, are known to be effective methods. For cognitive function, various neuropsychological tests are known according to the characteristics of the participants. Therefore, future studies may be needed to confirm the present findings and the benefits of casein-derived MKP on humans.

The present study showed the efficacy of casein-derived MKP intake on human BP and cognitive function. Furthermore, the safety of casein-derived MKP in humans was clarified. Casein-derived MKP may contribute to lowering SBP in the cardiovascular system and improving orientation in the brain. Based on these results, it is expected that the intake of casein-derived MKP extends healthy life expectancy in an aging society through the prevention of hypertension and dementia.

Appendices

Appendix A

Pancreatic Lipase Inhibitory Effect of Polyphenols Extracted From Black Tea Residue by Hot-Compressed Water

Abstract

Polyphenols, retained in black tea leaves following the commercial production of tea beverages, represent an underutilized resource. The purpose of this study was to investigate the potential use of hot-compressed water (HCW) for the extraction of polyphenols with pancreatic lipase inhibitory activity from black tea residue. Black tea residues were treated with HCW at 10°C intervals, from 100 to 200°C. The resulting extracts were analyzed using liquid chromatography-mass spectrometry and assayed to determine their inhibitory effect on pancreatic lipase activity *in vitro*. Four theaflavins, five catechins, two quercetin glycosides, quinic acid, gallic acid, and caffeine were identified. The total polyphenol content of extracts increased with increasing temperature. However, lipase inhibitors (theaflavin, theaflavin 3-*O*-gallate, theaflavin 3'-*O*-gallate, theaflavin 3,3'-*O*-gallate, epigallocatechin gallate and epicatechin gallate) decreased over 150°C. All extracts inhibited pancreatic lipase, but extracts obtained at 100–140°C showed the greatest lipase inhibition (IC_{50}s of 0.9–1.3 μg/ml). This is consistent with the optimal extraction of theaflavins and catechins except for catechin by HCW at near 140°C. HCW can be used to extract the polyphenols with pancreatic lipase inhibitory activity from black tea residue. These extracts have the potential to be used in dietary supplements and functional foods for the prevention and treatment of obesity.

A.1 Introduction

Tea is one of the most popular beverages in the world [1]. Black tea, prepared from the leaves of *Camellia sinensis*, accounts for the majority of tea consumed worldwide. Once the tea has been brewed, the used tea leaves become waste. Except for those used as agricultural feedstock

or turned into compost [2], these tea wastes are currently incinerated or sent to landfills. Researchers interested in reducing environmental load have investigated the potential uses of food wastes (e.g., as an energy source [3] or as active carbon [4,5]). However, the utilization of tea residues for the recovery of biologically active compounds remains relatively unexplored.

Tea leaves contain polyphenols, anti-oxidants with many beneficial biological properties [6,7]. Recently, polyphenols have received much attention for their anti-obesity effect. One effective way of preventing and treating obesity is to decrease fat absorption by the body. Ingested triacylglycerol forms micelles in the duodenum and must be converted into a fatty acid and 2-monoacylglycerol by pancreatic lipase, before absorption by epithelial cells in the small intestine [8]. Accordingly, inhibition of pancreatic lipase prevents or delays the adsorption of triacylglycerol [9,10]. Polyphenols found in black tea are known to have an inhibitory effect on pancreatic lipase [11,12]. If these substances can be recovered from black tea residues, they could be used in dietary supplements and functional foods.

Because tea brewing is a gentle process, many polyphenols are likely retained in the residues. However, there is little knowledge on the effective extraction methods. Several methods, that have been investigated for the extraction of compounds from unused food wastes, include supercritical water [13], sub-critical water [14,15], and hot-compressed water (HCW) [16]. Supercritical water involves heating water under pressure to a temperature and pressure above its critical point (372.4°C, 22.06 MPa). Under these conditions, the dielectric constant of the water becomes equivalent to those of organic solvents (e.g., chloroform and ethyl ether) and the dissociation constants for hydrogen and hydroxyl ions become higher than that of ambient water [17]. This method is expected to replace extraction with organic solvents. However, it

currently has a major disadvantage in that it requires the use of expensive, non-oxidizing material. Fortunately, sub-critical water and HCW can also be used for the extraction of compounds from food wastes [18]. In these conditions, it is difficult that the amount of tea residue is significantly reduced because the biopolymer such as cellulose and lignin are not degraded much [19]. However, the dielectric constant of HCW at 200°C is less than half that of ambient water (equivalent to that of methanol or ethylene glycol). Therefore, HCW has potential application for the extraction of useful substances from organic wastes, including tea residues.

The purpose of this study was to investigate the potential of HCW for the extraction of polyphenols with pancreatic lipase inhibitory activity from black tea residue. Black tea residues were extracted using HCW at 10°C intervals, from 100 to 200°C. Qualitative and quantitative analyses of extracts were conducted using liquid chromatography-mass spectrometry (LC-MS), and the inhibitory effect of the extracts, on pancreatic lipase, was tested *in vitro*.

A.2 Materials and Methods

A.2.1 Chemicals

Acetonitrile, water, and formic acid were of LC-MS grade (Wako Pure Chemical Industries Ltd., Osaka, Japan). Quinic acid, gallic acid, methyl gallate, ethyl gallate, caffeine, and 5-hydroxymethyl-2-furaldehyde (HMF) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Quercetin 3-*O*-glucoside and rutin trihydrate (as rutin) were purchased from Sigma Chemical Co. (St. Louis, MO). Theaflavin (TF), theaflavin 3-*O*-gallate (TF3G), theaflavin 3'-*O*-gallate (TF3′G), theaflavin 3,3'-*O*-gallate (TF3,3'G), (−)-epigallocatechin

(EGC), (−)-epigallocatechin gallate (EGCG), (−)-epicatechin gallate (ECG), (−)-epicatechin (EC), and (+)-catechin were purchased from Funakoshi Co. (Tokyo, Japan). Pancreatic lipase (type II, from the porcine pancreas; EC 3.1.1.3) and 4-methylumbelliferyl oleate were purchased from Sigma Chemical Co. Folin-Ciocalteu reagent and sodium carbonate were purchased from Wako Pure Chemical Industries Ltd.

A.2.2 Preparation of Black Tea Residue

Commercial black tea, a blended tea produced in Sri Lanka, was purchased in a local market. The black tea infusions were prepared by adding 20 volumes of boiling water to the leaves. The tea was allowed to steep for 6 min and was then filtered using a 42-mesh (0.355 mm pore size) sieve. The collected black tea leaves were compressed, to eliminate excess water, and lyophilized before conducting the experiments.

A.2.3 Hot-Compressed Water Treatment of Black Tea Residue

HCW treatments were performed with a portable pressure system (TPR-1-TVS-N-50; Taiatsu Techno, Tokyo, Japan) consisting of a reactor, an agitator, a pressure gauge, and two (internal and external) thermometers. The reactor had a maximum temperature and pressure ratings of 300°C and 20 MPa, respectively. Aliquots (21 g) of black tea residue were suspended in 300 mL of ion-exchange water (a liquid/solid ratio of 300:21) in the reactor, and the reactor was sealed. The pressure was not controlled but allowed to change as a function of temperature. The heating control was adjusted to obtain the desired temperatures, and cooling was initiated when the reactor reached these temperatures. When the internal temperature had fallen to 90°C, the

reactor was opened and the sample was collected. Samples were filtered with a 42-mesh (0.355 mm pore size) sieve to remove remained residue. The remaining residues were compressed to remove excess water, lyophilized, and weighed. The aqueous extract and liquid recovered from compression of remaining residues were combined and centrifuged (10000*g*, 20 min, 20°C). The supernatant was collected, filtered using filter paper (Qualitative filter paper No. 1, 125 mm; Toyo Roshi Kaisha, Tokyo, Japan), and the filtrate was lyophilized. The resulting powdered samples represented the black tea residue extracts. These were used for subsequent analyses.

This procedure was repeated at 10°C intervals for temperatures ranging from 100 to 200°C and triplicate samples were extracted at each temperature.

A.2.4 Preparation of Samples for LC-MS

One milligram of the powdered extract was dissolved in 1 ml ultra-pure water and then heated in a hot-water bath at 60°C. The dissolved samples were homogenized using an ultrasonicator (VS-01RD; Velvo-Clear, Tokyo, Japan), for 2 min, and then centrifuged (12000*g*, 10 min, 4°C). The supernatant was filtered through a 0.45 μm filter (HLC-DISK 25, 0.45 μm polysulphone; Kanto Chemical Co., Tokyo, Japan), and the filtrate was used for LC-MS analysis.

A.2.5 LC-MS Analysis

A Prominence semi-micro LC system (Shimadzu Co., Kyoto, Japan) coupled to an LTQ XL high-performance linear ion trap (Thermo Fisher Scientific K. K., Yokohama, Japan) with an electrospray interface was used for LC-MS analysis. The LC system comprised a system

controller (CBM-20A), degasser (DGU-20A3), pump (LC-20AD2), auto-sampler (SIL-20AC), UV/VIS detector (SPD-20A), and a column oven (CTO-20AC). The data were acquired using Xcalibur software (Version 1.4; Thermo Fisher Scientific). Samples were applied to a TSKgel ODS-100V, C18 reverse-phase column (5 μm particle size, 250 mm × 2.0 mm i.d.; TOSOH, Tokyo, Japan). Water (LC-MS grade; solvent A) and acetonitrile (LC-MS grade; solvent B), with 0.2% v/v formic acid added to each, were used as the mobile phases. The gradient program was as follows: 2% B (5 min), 2% B to 10% B (5 min), 10% B to 20% B (30 min), 20% B to 60% B (30 min), 60% B (5 min), 60% B to 2% B (1 min), and 2% B (10 min). The flow rate was set at 0.2 ml/min, and the column oven temperature was set at 40°C. Ten microliters of each sample were injected, and the absorbance at 280 nm was monitored.

Samples were analyzed using both negative and positive ionization modes. Electrospray ionization (ESI)-MS parameters included a spray voltage of 4.0 kV and capillary temperature of 300°C. Nitrogen sheath gas and auxiliary gas were set at 40 and 15 arbitrary units, respectively. A full-scan MS was performed in the m/z range 100–2000. The mass tolerance was set to 5 ppm. MS/MS fragmentations were carried out at normalized collision energy of 35.0%.

Quinic acid, gallic acid, methyl gallate, ethyl gallate, HMF, EGC, (+)-catechin, EC, EGCG, ECG, quercetin 3-*O*-glucoside, TF, TF3G, TF3'G, TF3,3'G, and caffeine were all quantified by reference to standard calibration curves, calculated using peak areas of base peak ion chromatograms. The standard calibration curves for each compound were as follows: quinic acid, $y = 0.000006x - 1.7278$; gallic acid, $y = 0.000005x + 0.3269$; methyl gallate, $y = 0.000002x - 0.2344$; ethyl gallate, $y = 0.000002x - 0.0026$; HMF, $y = 0.000005x + 0.0922$;

EGC, y = 0.000004x – 0.0097; (+)-catechin, y = 0.000004x – 0.1607; EC, y = 0.000002x – 0.0584; EGCG, y = 0.000004x + 0.0788; ECG, y = 0.000002x – 0.0492; quercetin 3-*O*-glucoside, y = 0.000001x + 0.2067; TF, y = 0.000003x + 0.3188; TF3G, y = 0.000003x + 0.3873; TF3'G, y = 0.000004x + 0.3913; TF3,3'G, y = 0.000002x + 0.4733; caffeine, y = 0.000003x – 1.4184. The compounds in the extracts were identified by comparison of their retention time, absorbance spectrum, and MS fragmentation and chromatography patterns, with the above standards.

A.2.6 Measurement of Pancreatic Lipase Activity

The enzymatic activity of pancreatic lipase was assayed using a modification of the previously described method [20]. Twenty-five microliters of aqueous black tea residue extracts, or purified compounds (orlistat, TF, TF3G, TF3'G, TF3,3'G, EGC, EGCG, ECG, EC, (+)-catechin, quinic acid, gallic acid, caffeine, quercetin 3-*O*-glucoside, and rutin) and 50 µl of 0.1 mM 4-methylumbelliferyl oleate solution (in 13 mM Tris-HCl, 150 mM NaCl, and 1.3 mM $CaCl_2$ (pH 8.0)) were mixed in the well of a microtiter plate, and 25 µl of the lipase solution (400 U/ml in the above buffer) was added to start the enzyme reaction. After incubation at 25°C for 30 min, 100 µl of 0.1 M sodium citrate (pH 4.2) was added to stop the reaction. The amount of 4-methylumbelliferone released by the lipase was measured with a microplate reader (SH-9000; Corona Electric Co., Ibaraki, Japan) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The IC_{50} of the test sample was obtained from the least-squares regression line of the plots of the logarithm of the sample concentration versus the pancreatic lipase activity.

A.2.7 Measurement of Total Polyphenol

To quantify the total polyphenol concentration in samples, a modification of the Folin-Denis method was applied, and the data were expressed in gallic acid equivalents [21]. One hundred microgram aliquots of the powdered samples were dissolved in 1 ml of deionized water, and 0.25 ml of the solution was added to a test tube. Deionized water was used as a reagent blank. Folin-Ciocalteu reagent (1.25 ml) was diluted with 2 volume of deionized water, and 1.25 ml of 10% sodium carbonate were added to the test tube and mixed. After incubation at 25°C for 20 min, the absorbance, against the reagent blank, was determined at 760 nm using a microplate reader (SH-9000). Three replicate samples were examined and SDs were calculated.

A.3 Results and Discussion

A.3.1 Hot-Compressed Water Treatment of Black Tea Residue

Black tea residues were treated with HCW under different physical conditions. As the temperature of the HCW increased, the turnaround time (total incubation time) also increased. At 100°C, the minimum turnaround time (5.23 ± 0.68 min) was observed and the maximum turnaround time (26.67 ± 0.36 min) was observed at 200°C (Table A-1). In contrast, as temperature and turnaround time increased, the theoretical pKw and theoretical dielectric constants decreased. The dielectric constant at 100°C was 55.53, but decreased to 34.75 (similar to methanol) at 200°C (Table A-1). These changes are reflected in the amount of total extract and remaining residues recovered from HCW extracts. Total extracts increased with increasing temperature (decreasing dielectric constant) while remaining residues decreased (Figure A-1). The sum of the amounts of extracts and remaining residues should be about 1.0 μg/g tea residue.

However, the data were much less than 1.0 μg/g tea residue. Two reasons were considered. One may be the loss in operations such as filtration and the other may be the loss of volatile contents. The minimum total extract (0.05 g/g tea residue) was observed at 100°C, while the maximum total extract (0.21 g/g tea residue) was achieved at 200°C (Figure A-1). These results were supported by reversed-phase high-performance liquid chromatography (HPLC) chromatograms. Reversed-phase HPLC of extracts obtained at 100, 140, and 180°C showed increasing yield with increasing temperature (Figure A-2). On the other hand, even at 200°C, more than half of the tea residue remained after HCW treatment. Black tea residue involves cellulose, hemicellulose, lignin, and other minor components such as minerals, aliphatic compounds, proteins, and phenolic compounds [19]. In these components, especially lignocellulosic biopolymers such as cellulose and lignin may be difficult to be decomposed. Accordingly, complete decomposition of the tea residue by only a simple hydrothermal reaction is difficult. For example, although physical force such as shear and/or pressure contributes to decreasing the amount of remained tea residue, complete decomposition may not be achieved. Therefore, the remaining tea residue should be used for other applications such as solid fuel, filtration agent, building material, and so on.

Table A-1 Conditions of HCW treatment at each temperature.

Temperature (°C)	Time (min)	Actual pressure (MPa)	Theoretical pressure (MPa)	Theoretical pKw	Theoretical relative dielectric constant
100	5.23 ± 0.68	0.1	0.102	12.04	55.53
110	5.53 ± 0.45	0.1	0.145	11.86	53.02
120	6.88 ± 0.62	0.2	0.202	11.69	50.62
130	7.72 ± 0.54	0.3	0.276	11.54	48.33
140	7.78 ± 0.43	0.4	0.370	11.40	46.13
150	9.88 ± 0.65	0.5	0.490	11.28	44.03
160	13.00 ± 0.38	0.6	0.640	11.16	42.02
170	14.88 ± 0.56	0.8	0.824	11.06	40.09
180	15.45 ± 0.45	1.0	1.049	10.96	38.24
190	20.00 ± 0.78	1.2	1.320	10.88	36.46
200	26.67 ± 0.36	1.5	1.644	10.81	34.75

Data represent mean ± SD (n = 3).

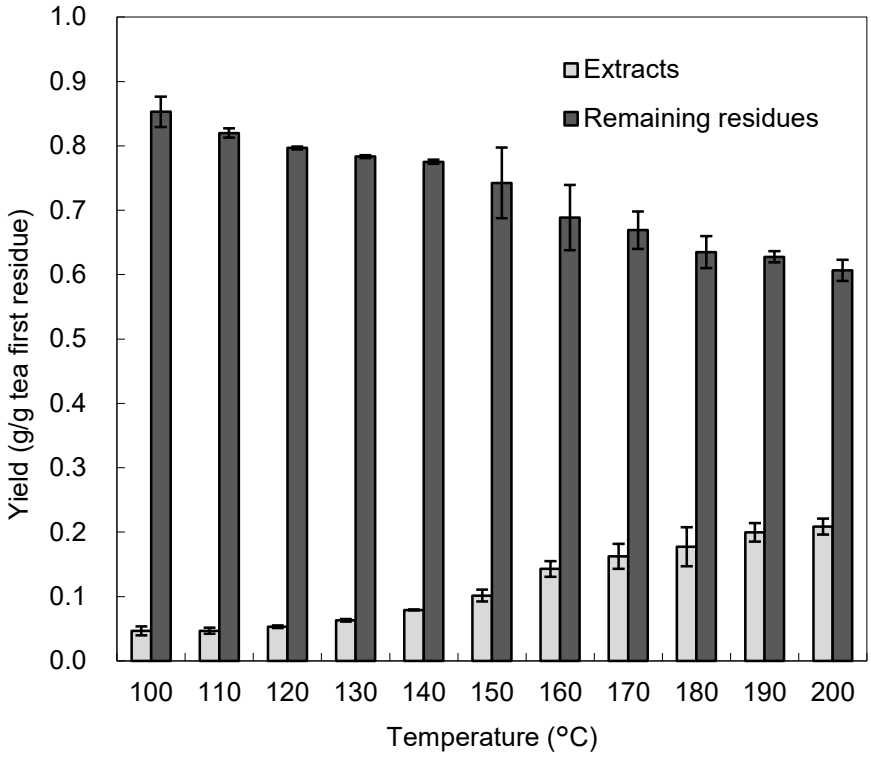

Figure A-1 Yields of extracts and remaining residues from black tea residue using HCW at each temperature.

Error bars indicate SD (n = 3).

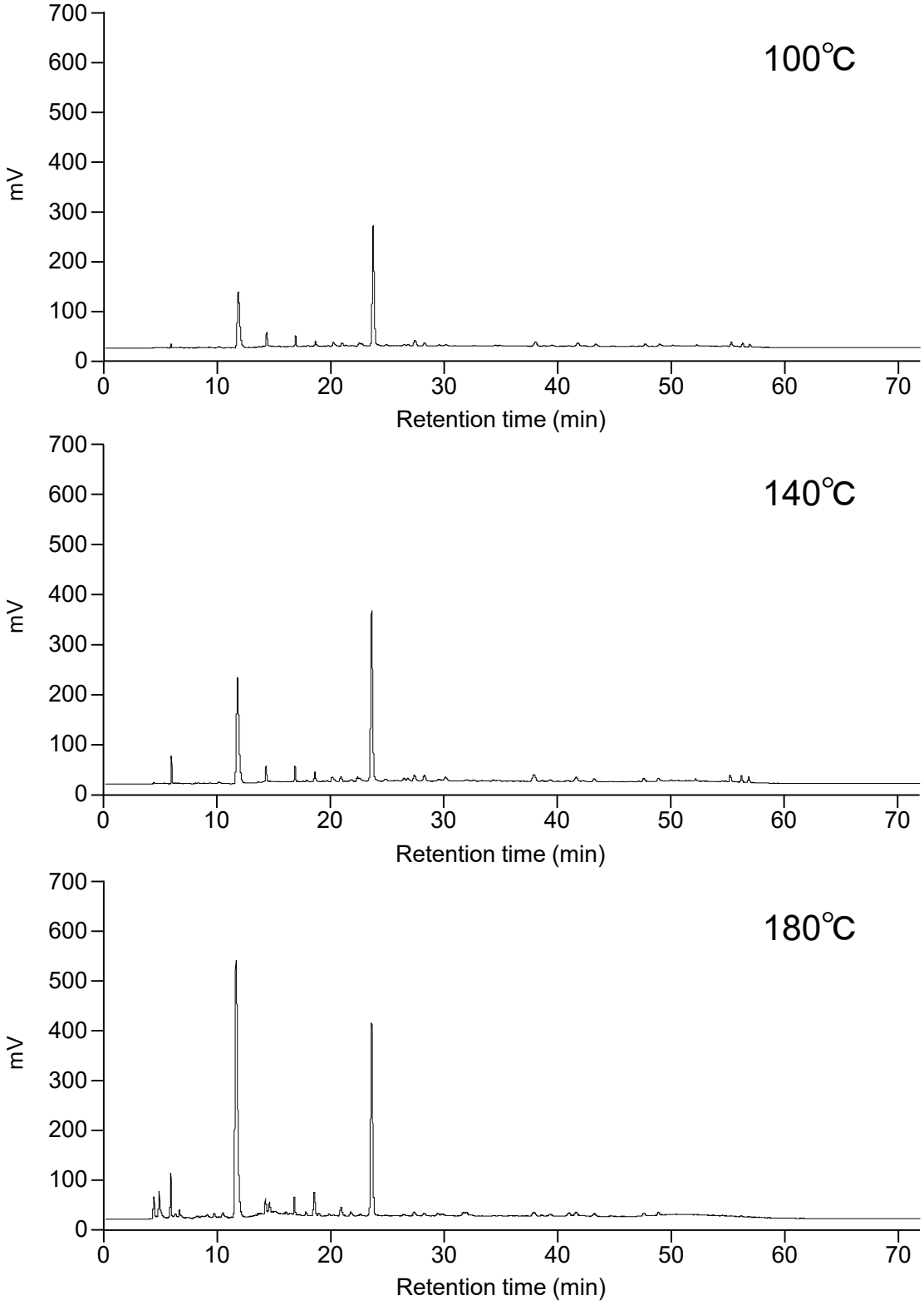

Figure A-2 Reversed-phase HPLC analysis of extracts from black tea residue using HCW at 100, 140, and 180°C.

A.3.2 LC-MS Analysis

LC-MS analysis identified 14 peaks. More peaks were detected by ESI negative ion mode than by positive ion mode (Figures A-3 and A-4). Comparison of retention times, m/z, and MS/MS identified these peaks as quinic acid (4.8 min), gallic acid (11.8 min), EGC (20.1 min), (+)-catechin (21.8 min), caffeine (23.4 min), EC (26.4 min), EGCG (28.3 min), ECG (37.9 min), rutin (41.0 min), quercetin-3-*O*-glucoside (43.1 min), TF (54.6 min), TF3G (55.7 min), TF3'G (56.2 min), and TF3,3'G (56.3 min) (Table A-2; for chemical structures see Figure A-5).

LC-MS was also used to quantify the yields of individual compounds present in the extracts obtained at all experimental temperatures (Table A-3). As the treatment temperature increased, yields of quinic acid, gallic acid, and caffeine also increased. The maximum amount of quinic and gallic acids were observed at 180 and 190°C, respectively. Caffeine continued to increase up to 200°C. The amount of rutin and quercetin 3-*O*-glucoside did not change much from 100 to 200°C. Optimal recovery of TF, TF3G, TF3'G, TF3,3'G, EGC, EGCG, ECG, and EC was observed between 130 and 150°C, but (+)-catechin yield was greatest at 190°C. Methyl gallate, ethyl gallate, and HMF were not detected.

The inversely proportional response of the acids (quinic and gallic acids) and the theaflavins and catechins to increasing temperature indicates that hydrolysis of the latter increased under conditions associated with elevated HCW temperatures. Although some free quinic acid is present in black tea leaves, a large portion occurs as theogallin, a compound formed by the coupling of gallic acid and quinic acid [7,22]. Hydrolysis of theogallin releases quinic acid. In addition, gallic acid occurs as hydrolytic tannin including catechins, theaflavins, and thearubigins. Therefore, hydrolysis of these compounds also produces gallic acid.

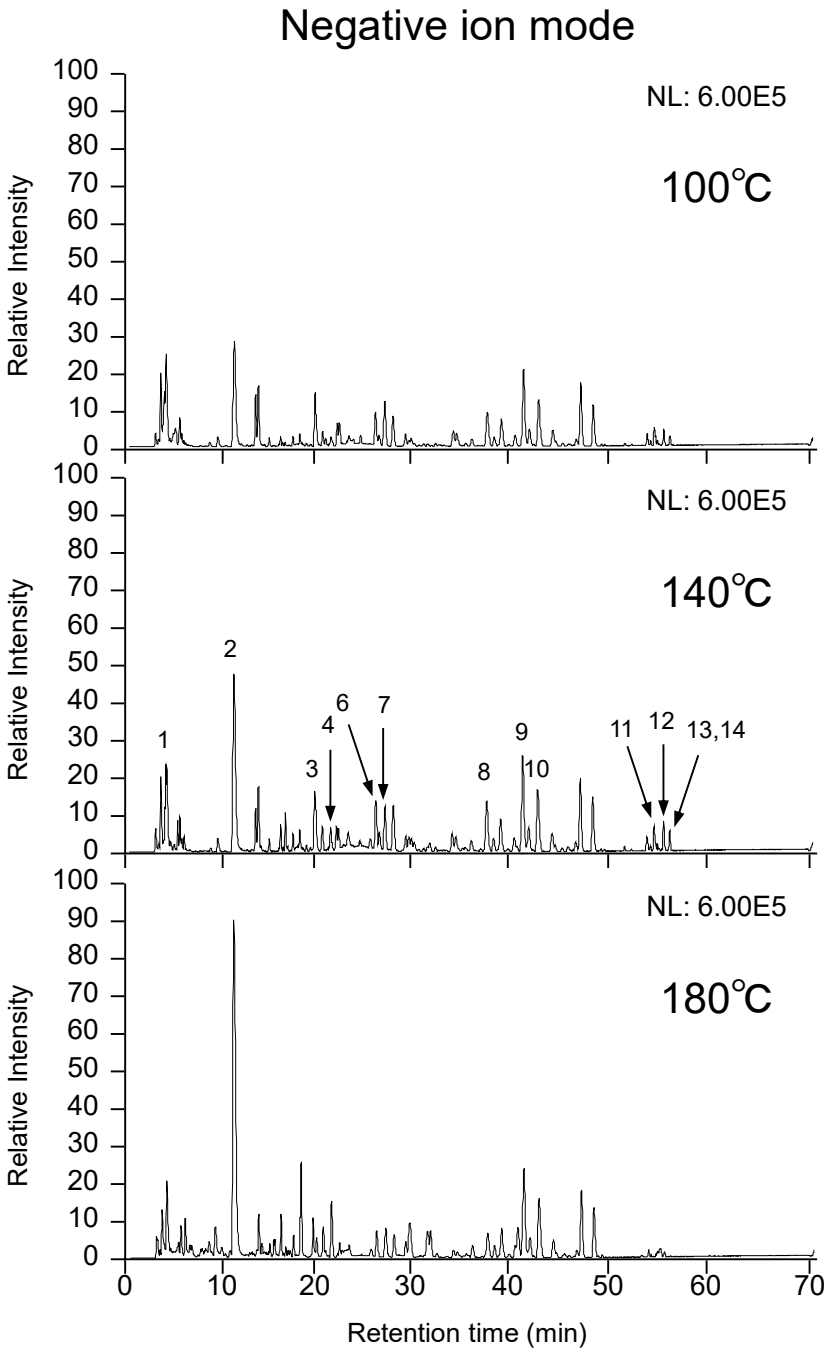

Figure A-3 LC-MS analysis of extracts from black tea residue using HCW at 100, 140, and 180°C in negative ion mode.

Concentrations of each sample were 0.22% (100°C), 0.38% (140°C), and 0.85% (180°C) corresponding to the yields of extracts from 5 g of black tea residue at each temperature. Peaks annotations match those in Table A-2.

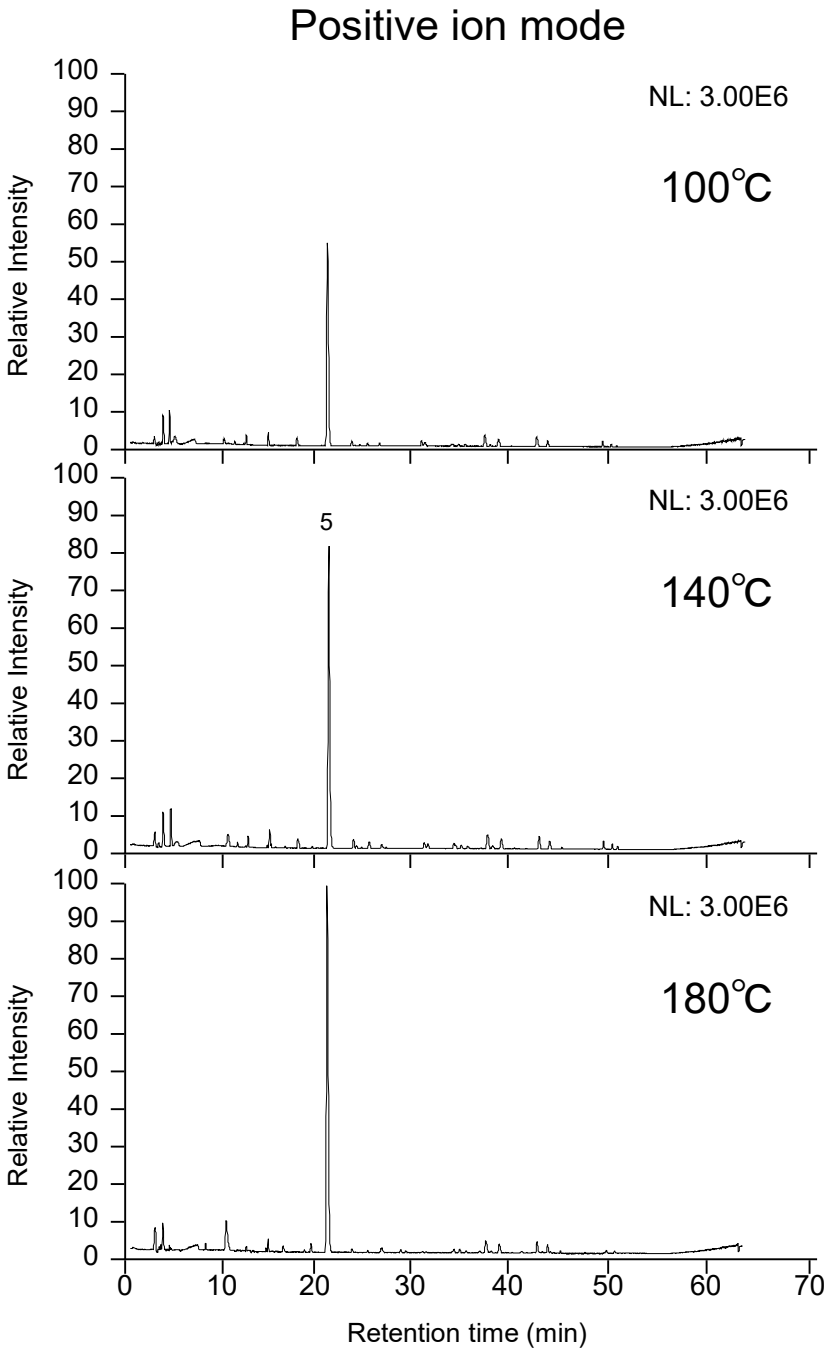

Figure A-4 LC-MS analysis of extracts from black tea residue using HCW at 100, 140, and 180°C in positive ion mode.

Concentrations of each sample were 0.22% (100°C), 0.38% (140°C), and 0.85% (180°C) corresponding to the yields of extracts from 5 g of black tea residue at each temperature. Peaks annotations match those in Table A-2.

Table A-2 Identified compounds in extracts from black tea residue.

Peak	t_R (min)	Compound	$[M-H]^-$ (m/z)	MS/MS (m/z)				
1	4.8	Quinic acid	191	173	127	93	85	
2	11.8	Gallic acid	169	125				
3	20.1	(−)-Epigallocatechin	305	261	221	219	179	165
4	21.8	(+)-Catechin	289	245	205	203	179	
5	23.4	Caffeine[a]	195	138				
6	26.4	(−)-Epicatechin	289	245	205	203	179	
7	28.3	(−)-Epigallocatechin gallate	457	331	305	193	169	
8	37.9	(−)-Epicatechin gallate	442	331	289	272	170	
9	41.0	Rutin	610	343	301	300		
10	43.1	Quercetin 3-*O*-glucoside	463	301	300			
11	54.6	Theaflavin	563	545	407	379	241	
12	55.7	Theaflavin 3-*O*-gallate	716	563	545	527	501	483
13	56.2	Theaflavin 3'-*O*-gallate	716	577	563	545	407	69
14	56.3	Theaflavin 3,3'-*O*-gallate	868	715	697	545	527	389

Peak numbers and retention times refer to Figures A-3 and A-4. [a] $[M+H]^+$.

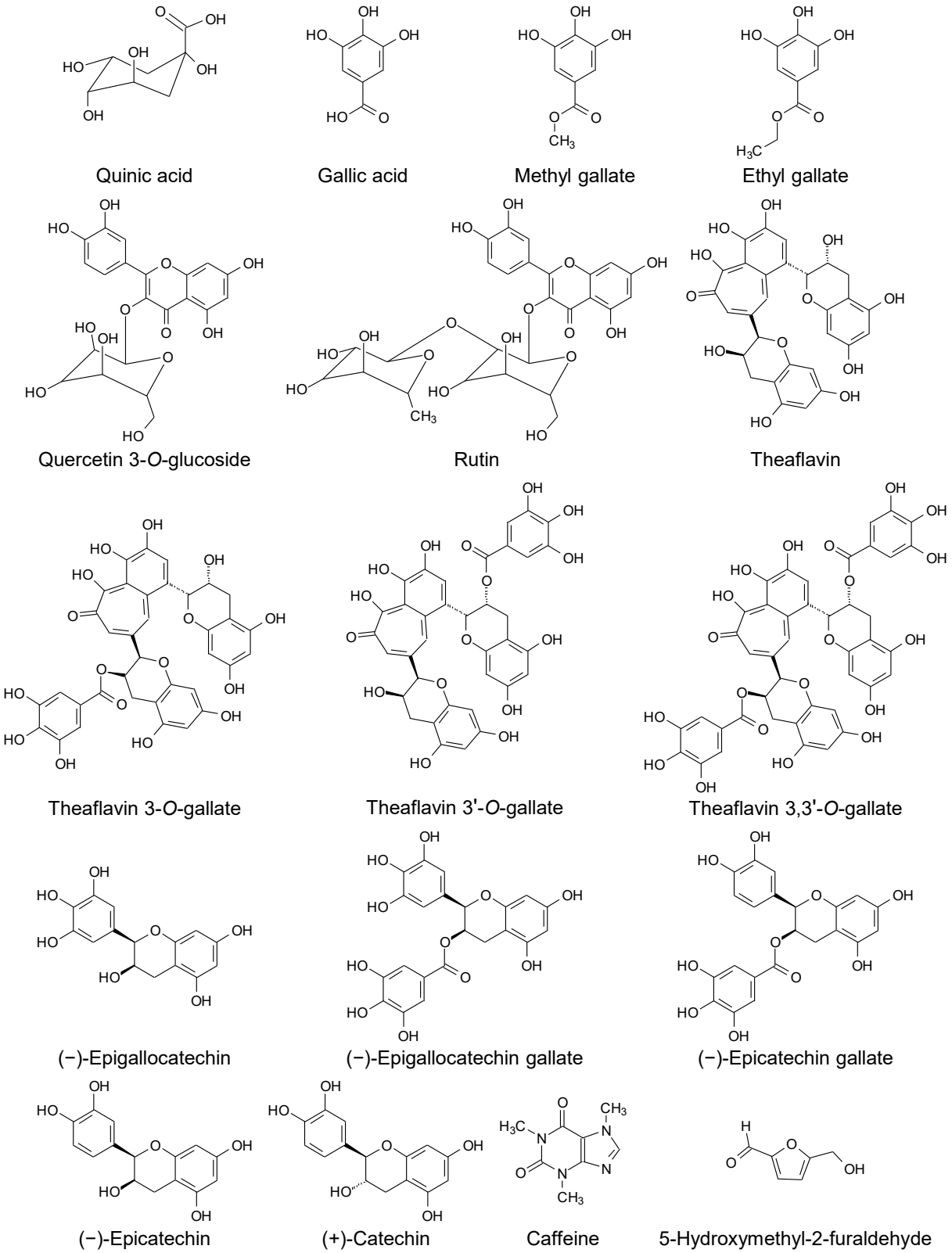

Figure A-5 Structures of compounds.

Table A-3 Quantity of compounds in extracts from black tea residue.

No.	Compound	Temperature (°C)										
		100	110	120	130	140	150	160	170	180	190	200
		mg/g of black tea residue										
1	Quinic acid	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	1.4 ± 0.1	1.8 ± 0.1	2.1 ± 0.1	2.1 ± 0.1	2.0 ± 0.1	1.6 ± 0.2
2	Gallic acid	0.7 ± 0.0	0.7 ± 0.0	0.9 ± 0.0	1.2 ± 0.1	1.7 ± 0.1	2.4 ± 0.1	3.0 ± 0.1	3.6 ± 0.2	4.2 ± 0.2	5.6 ± 0.2	5.5 ± 0.2
3	(−)-Epigallocatechin	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.0 ± 0.0
4	(+)-Catechin	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.1 ± 0.0
5	Caffeine	2.9 ± 0.1	3.1 ± 0.2	3.4 ± 0.1	3.7 ± 0.1	4.1 ± 0.2	3.8 ± 0.0	4.0 ± 0.0	4.4 ± 0.1	4.6 ± 0.1	5.5 ± 0.0	5.7 ± 0.1
6	(−)-Epicatechin	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.0 ± 0.0
7	(−)-Epigallocatechin gallate	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.0 ± 0.0
8	(−)-Epicatechin gallate	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.0 ± 0.0
9	Rutin	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.2 ± 0.0
10	Quercetin 3-*O*-glucoside	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
11	Theaflavin	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	ND
12	Theaflavin 3-*O*-gallate	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	ND
13	Theaflavin 3'-*O*-gallate	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	ND	ND
14	Theaflavin 3,3'-*O*-gallate	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	ND

Data represent mean ± SD (n = 3). ND, not detected.

A.3.3 Pancreatic Lipase Inhibition

Temperature-dependent differences in the extraction of these various compounds influenced the inhibitory effects of extracts on pancreatic lipase. Black tea residue extracts treated with HCW between 100 and 140°C had the greatest inhibitory effect on pancreatic lipase with IC_{50} values of 0.9–1.3 μg/mL (Figure A-6). As the HCW treatment temperature was increased over 150°C, the extracts displayed lower inhibitory activity. These results were consistent with the optimal extraction of theaflavins and catechins (except (+)-catechin) observed at near 140°C. In this assay, theaflavins (TF, TF3G, TF3'G, and TF3,3'G) and the catechins (EGCG and ECG) showed a relatively strong ability to inhibit pancreatic lipase (IC_{50} values ranging from 0.081 to 1.046 μg/mL), compared to the positive control (orlistat; IC_{50} = 0.042 μg/mL) (Table A-4). In contrast, EGC, gallic acid, quercetin-3-*O*-glucoside, and rutin showed very weak activity (IC_{50} values > 50 μg/mL), and activity was not detected in EC, (+)-catechin, quinic acid, caffeine, methyl gallate, ethyl gallate, and HMF (Table A-4). It is known that polyphenols derived from green tea, oolong tea, and black tea have inhibitory effects on pancreatic lipase [11,12]. However, polyphenols such as EC and (+)-catechin did not show the ability to inhibit pancreatic lipase.

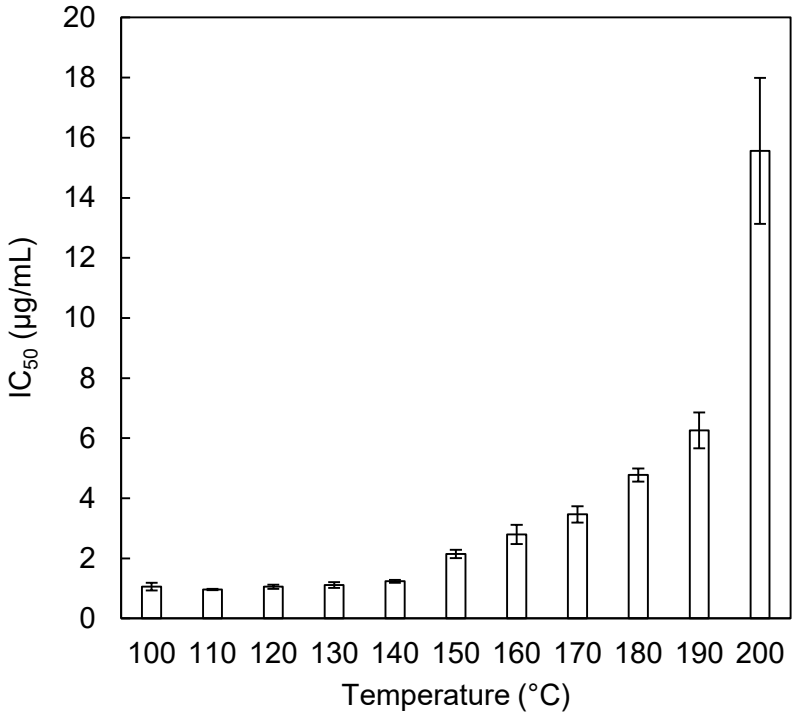

Figure A-6 Inhibitory effects of HCW extracts from black tea residue on pancreatic lipase. Error bars indicates SD (n = 3).

Table A-4 Inhibitory effects of compounds on pancreatic lipase.

Compound	IC_{50} (ug/mL)
Orlistat	0.042
Theaflavin	0.679
Theaflavin 3-*O*-gallate	0.368
Theaflavin 3'-*O*-gallate	0.320
Theaflavin 3,3'-*O*-gallate	0.316
(−)-Epigallocatechin	39.192
(−)-Epigallocatechin gallate	0.081
(−)-Epicatechin gallate	1.046
(−)-Epicatechin	ND
(+)-Catechin	ND
Quinic acid	ND
Gallic acid	> 50
Caffeine	ND
Quercetin 3-*O*-glucoside	> 50
Rutin	> 50
Methyl gallate	ND
Ethyl gallate	ND
5-Hydroxymethyl-2-furaldehyde	ND

ND, not detected.

A.3.4 Polyphenols With Pancreatic Lipase Inhibition

From the results of Table A-4, theaflavins, EGCG, and ECG were treated as lipase inhibitors from black tea residue in this study. Comparison of the yields of total polyphenols and lipase inhibitors from black tea residue indicated that despite the increasing yields of total polyphenols with increasing temperature, HCW at near 140°C was optimal for the extraction of pancreatic lipase inhibitory polyphenols (Figure A-7). Figure A-8 shows the proportion of either theaflavins, EGCG or ECG to the total amount of polyphenols. The yields of compounds with

the greatest inhibitory activity (theaflavins, EGCG and ECG) all decreased with increasing temperature of over 150°C (Figure A-8). It is considered that the presence of galloyl moieties within their chemical structures and/or the polymerization of flavan-3-ols were required for enhancement of pancreatic lipase inhibition [11,23]. From the results of LC-MS analysis, gallic acid increased, and condensed tannin such as theaflavins, EGC, EGCG, and ECG decreased with increasing treatment temperature. These results suggest that HCW treatments over 150°C have a negative effect on pancreatic lipase inhibition by extracts from the black tea residue because gallic moieties were hydrolyzed. A further important point was that extractions from the black tea residue with HCW at near 140°C may have a positive impact on the yields of lipase inhibitors.

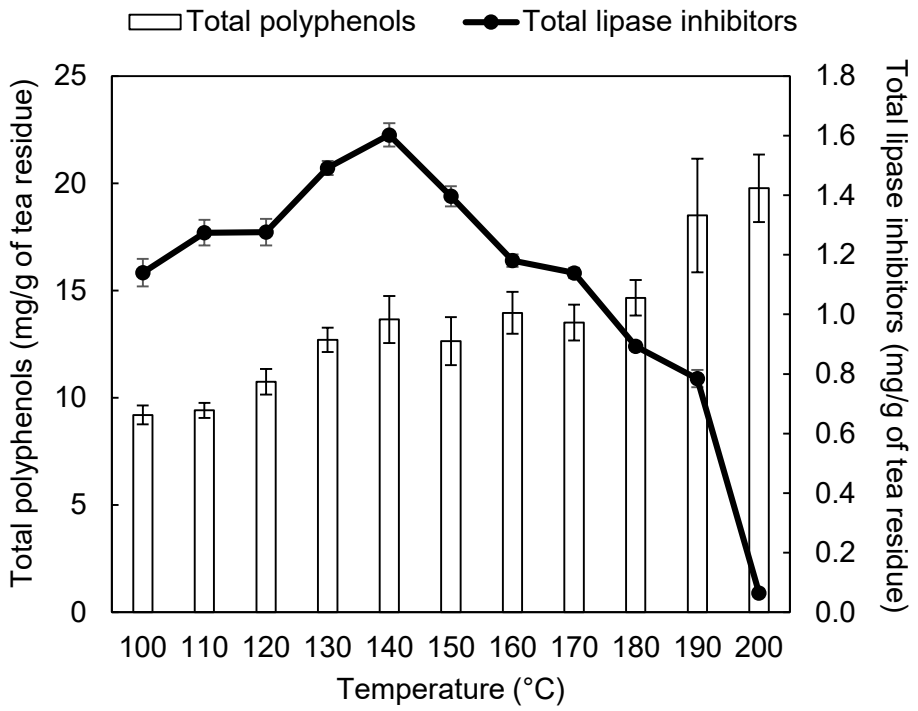

Figure A-7 Total polyphenols and total lipase inhibitors of HCW extracts from black tea residue.

Error bars indicate SD (n = 3).

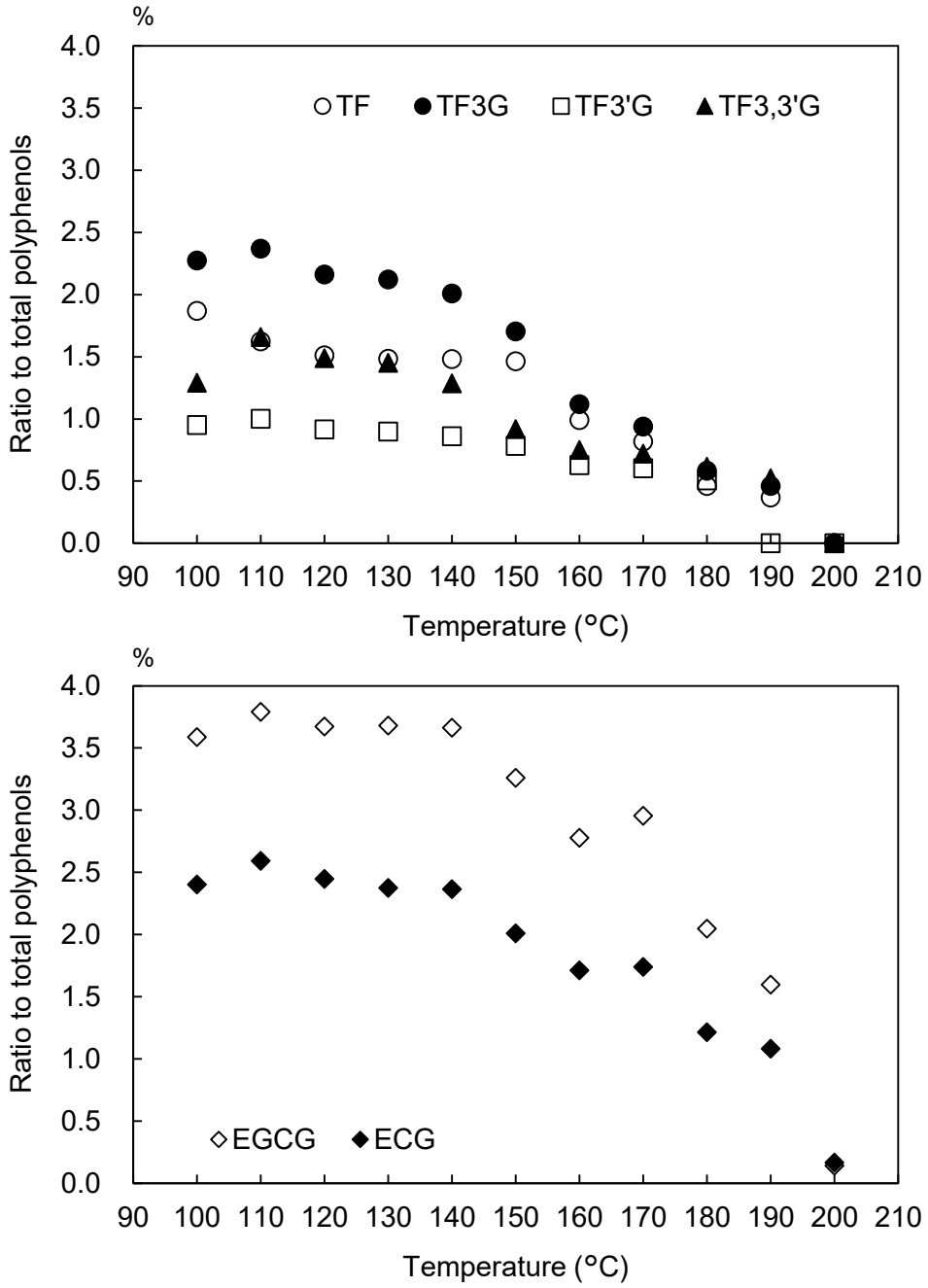

Figure A-8 The ratio of TF, TF3G, TF3'G, TF3,3'G, EGCG, and ECG to total polyphenols in HCW extracts of black tea residue.

A.4 Conclusions

This study demonstrates that HCW can be used to extract the polyphenols with pancreatic lipase inhibitory activity from black tea residue. These extracts have the potential to be used in dietary supplements and functional foods for the prevention and treatment of obesity. The optimal temperature for the extraction of these compounds was near 140°C. Over 150°C, hydrolysis of theaflavins and catechins appeared to increase, resulting in a greater yield of polyphenols but a decrease in the yield of active pancreatic lipase inhibitors.

It is known that the extraction heating process contributes to the quantity of polyphenol. However, this study clarified the fact that extraction heating did not always contribute to the quality of polyphenol, especially related to lipase inhibition. This fact indicates that the extraction heating may affect other physiological features of the polyphenols, such as anti-oxidative activities. On the other hand, if the extraction is executed at an appropriate temperature, valuable products are recovered even from the residues. Dehydration of black tea residue on an industrial scale may be difficult because of its higher moisture content than that of other residues, such as coffee. Therefore, the burning of black tea residue takes enormous energy. In the present set of circumstances, black tea residue is often discarded as industrial waste except for utilization as feed or compost. The present study suggests that HCW treatment of black tea residue at a certain temperature range may have the potential to extract the polyphenols without loss of its functions.

References

[1] van der Burg-Koorevaar MC, Miret S, Duchateau GS. Effect of milk and brewing method on black tea catechin bioaccessibility. *J Agric Food Chem*. 2011;59(14):7752–7758. doi:10.1021/jf2015232

[2] Arvanitoyannis IS, Varzakas TH. Vegetable waste treatment: comparison and critical presentation of methodologies. *Crit Rev Food Sci Nutr*. 2008;48(3):205–247. doi:10.1080/10408390701279798

[3] Nakahara K, Kawabata S, Ono H, et al. Inhibitory effect of oolong tea polyphenols on glycosyltransferases of mutans Streptococci. *Appl Environ Microbiol*. 1993;59(4):968–973. doi:10.1128/AEM.59.4.968-973.1993

[4] Duran C, Ozdes D, Gundogdu A, Imamoglu M, Senturk HB. Tea-industry waste activated carbon, as a novel adsorbent, for separation, preconcentration and speciation of chromium. *Anal Chim Acta*. 2011;688(1):75–83. doi:10.1016/j.aca.2010.12.029

[5] Kante K, Nieto-Delgado C, Rangel-Mendez JR, Bandosz TJ. Spent coffee-based activated carbon: specific surface features and their importance for H_2S separation process. *J Hazard Mater*. 2012;201–202:141–147. doi:10.1016/j.jhazmat.2011.11.053

[6] Sharma V, Rao LJ. A thought on the biological activities of black tea. *Crit Rev Food Sci Nutr*. 2009;49(5):379–404. doi:10.1080/10408390802068066

[7] Drynan JW, Clifford MN, Obuchowicz J, Kuhnert N. The chemistry of low molecular weight black tea polyphenols. *Nat Prod Rep*. 2010;27(3):417–462. doi:10.1039/b912523j

[8] Lowe ME. Pancreatic triglyceride lipase and colipase: insights into dietary fat digestion. *Gastroenterology*. 1994;107(5):1524–1536. doi:10.1016/0016-5085(94)90559-2

[9] Hill JO, Hauptman J, Anderson JW, et al. Orlistat, a lipase inhibitor, for weight maintenance after conventional dieting: a 1-y study. *Am J Clin Nutr*. 1999;69(6):1108–1116. doi:10.1093/ajcn/69.6.1108

[10] Gu Y, Hurst WJ, Stuart DA, Lambert JD. Inhibition of key digestive enzymes by cocoa extracts and procyanidins. *J Agric Food Chem*. 2011;59(10):5305–5311. doi:10.1021/jf200180n

[11] Nakai M, Fukui Y, Asami S, et al. Inhibitory effects of oolong tea polyphenols on pancreatic lipase *in vitro*. *J Agric Food Chem*. 2005;53(11):4593–4598. doi:10.1021/jf047814+

[12] Kusano R, Andou H, Fujieda M, Tanaka T, Matsuo Y, Kouno I. Polymer-like polyphenols of black tea and their lipase and amylase inhibitory activities. *Chem Pharm Bull (Tokyo)*. 2008;56(3):266–272. doi:10.1248/cpb.56.266

[13] Jin FM, Kishita A, Moriya T, Enomoto H. Kinetics of oxidation of food wastes with H_2O_2 in supercritical water. *J Supercrit Fluids*. 2001;19(3):251–262. doi:10.1016/S0896-8446(00)00094-2

[14] Farhoosh R, Golmovahhed GA, Khodaparast MH. Antioxidant activity of various extracts of old tea leaves and black tea wastes (*Camellia sinensis* L.). *Food Chem*. 2007;100(1):231–236. doi:10.1016/j.foodchem.2005.09.046

[15] Wiboonsirikul J, Adachi S. Extraction of functional substances from agricultural products or by-products by subcritical water treatment. *Food Sci Technol Res*. 2008;14(4):319–319. doi:10.3136/fstr.14.319

[16] Etoh H, Ohtaki N, Kato H, Kulkarni A, Morita A. Sub-critical water extraction of residual green tea to produce a roasted green tea-like extract. *Biosci Biotechnol Biochem*. 2010;74(4):858–860. doi:10.1271/bbb.90800

[17] Marshall WL, Franck EU. Ion product of water substance, 0–1000 °C, 1–10,000 bars New International Formulation and its background. *J Phys Chem Ref Data*. 1981;10(2):295–304. doi:10.1063/1.555643

[18] Herrero M, Cifuentes A, Ibañez E. Sub-and supercritical fluid extraction of functional ingredients from different natural sources: plants, food-by-products, algae and microalgae: a review. *Food Chem*. 2006;98(1):136–148. doi:10.1016/j.foodchem.2005.05.058

[19] Tsubaki S, Iida H, Sakamoto M, Azuma J. Microwave heating of tea residue yields polysaccharides, polyphenols, and plant biopolyester. *J Agric Food Chem*. 2008;56(23):11293–11299. doi:10.1021/jf802253s

[20] Kurihara H, Asami S, Shibata H, Fukami H, Tanaka T. Hypolipemic effect of *Cyclocarya paliurus* (Batal) Iljinskaja in lipid-loaded mice. *Biol Pharm Bull*. 2003;26(3):383–385. doi:10.1248/bpb.26.383

[21] Folin O, Denis W. A colorimetric method for the determination of phenols (and phenol derivatives) in urine. *J Biol Chem*. 1915;22(2):305–308. doi:10.1016/S0021-9258(18)87648-7

[22] Del Rio D, Stewart AJ, Mullen W, et al. HPLC-MSn analysis of phenolic compounds and purine alkaloids in green and black tea. *J Agric Food Chem*. 2004;52(10):2807–2815. doi:10.1021/jf0354848

[23]Kajimoto O, Kajimoto Y, Yabune M, et al. Tea catechins with a galloyl moiety reduce body weight and fat. *J Health Sci*. 2005;51(2):161–171. doi:10.1248/jhs.51.161

Appendix B

Understanding the Foods With Function Claims System Through the Analytical Results of Notification Status

Abstract

The Foods with Function Claims (FFC) system was introduced in April 2015 to help consumers make more informed choices. One of the features of this system is that if the required information for a food product is notified to the Secretary-General of the Japanese Consumer Affairs Agency (CAA) before the product is marketed, the product is allowed to bear function claims stating the product's specified health benefit, under the food business operator's responsibility. Most of the product information submitted to the CAA is disclosed on the CAA website, which is also a unique feature of this system. As of March 31, 2017, the total number of FFC was 815, excluding 8 products that were withdrawn. In this review, using the information available on the CAA website, the number of products, food business operators, food categories, scientific evidence, functional substances, and function claims were analyzed. The results indicated that the FFC market is expanding. The current situation was examined based on the results of this analysis, and the future development of FFC was discussed.

B.1 Introduction

The Foods with Function Claims (FFC) are the third type of the Foods with Health Claims, following the Foods for Specified Health Uses (FOSHU) and the Foods with Nutrient Function Claims (FNFC). FOSHU requires that each product be reviewed for efficacy and safety, and that the labeling be approved by the Secretary-General of the Japanese Consumer Affairs Agency (CAA). Therefore, FOSHU has the advantage of being able to show labeling that has been authorized by the government. However, it has been difficult for small and medium-sized companies to apply for the FOSHU system because of the enormous cost and time required to

obtain scientific evidence on the efficacy and safety of their products. On the other hand, FNFC has the merit of being able to be labeled on food products without notification if the designated nutrient content conforms to the standard values. However, it is limited to the labeling of specific nutritional functions. To overcome these issues and enable consumers to make more informed choices, the FFC system was introduced in April 2015.

The FFC system provides opportunities for consumers to make voluntary and reasonable product choices based on the three concepts of 1) ensuring safety, 2) establishing requirements for scientific evidence to make function claims, and 3) providing consumers with information through proper labeling [1]. The most distinctive feature of this system is that food products can be labeled with functional properties if the necessary information is submitted to the CAA at least 60 days before sale. The necessary information includes details of the product labeling, basic information on food business operators such as name and contact information, scientific evidence of safety and effectiveness, information on the production, manufacturing and quality control system, the system to collect adverse health events, and other required information [2]. The CAA will check the information, and if it meets the requirements for notification, a notification number will be issued and the information will be disclosed in the database of FFC on the CAA website, which is also a unique feature of this system.

The FFC system has high expectations from consumers, food business operators, and the government. For consumers, it has the advantage of making it possible to learn about specific physiological functions in easy-to-understand terms and providing them with the opportunity to select appropriate products that suit them. For food business operators, if they obtain appropriate scientific evidence and meet the requirements of the system, they will have the

advantage of being able to promote their products in the field of physiological functions, which has not been possible before. For the government, there is the advantage of contributing to the maintenance and promoting the health of the people, as well as promoting the Japanese economy by expanding the food market. In addition, FFC also covers fresh foods, which may lead to an increase in high-value-added agricultural, forestry and fishery products. On the other hand, FFC imposes important responsibilities on food business operators. Unlike FOSHU, FFC is not individually approved by the government. Therefore, food business operators themselves must take responsibility for the contents of the notification, including the validity of scientific evidence.

As of March 31, 2017, the total number of FFC was 815, excluding 8 products that were withdrawn. In this review, using the information available on the CAA website [3], the number of products, food business operators, food categories, scientific evidence, functional substances, and function claims were analyzed. The current situation was examined based on the results of these analyses, and the future development of FFC was discussed.

B.2 Number of Products

The number of FFC was counted over time based on the disclosure date of notification (Figure B-1). In the first year of the system (April 2015–March 2016), the number of products increased at a pace of about 22 products/month. In the second year (April 2016–March 2017), the number of products increased to about 46 products/month, approximately doubling the pace of increase. Thus, the number of FFC is rapidly expanding. The FOSHU system was introduced in 1991, and over the past 26 years, 1127 products (as of March 9, 2017) have been approved. Therefore,

the number of FOSHU is likely to surpass that of FFC soon. The market size of FOSHU in 2015 was 639.1 billion Japanese yen (JPY), and the market for FFC was reported to be 44.6 billion JPY in 2015 and is expected to be 148.3 billion JPY in 2016. Therefore, the market size of FFC is expected to expand further in the future.

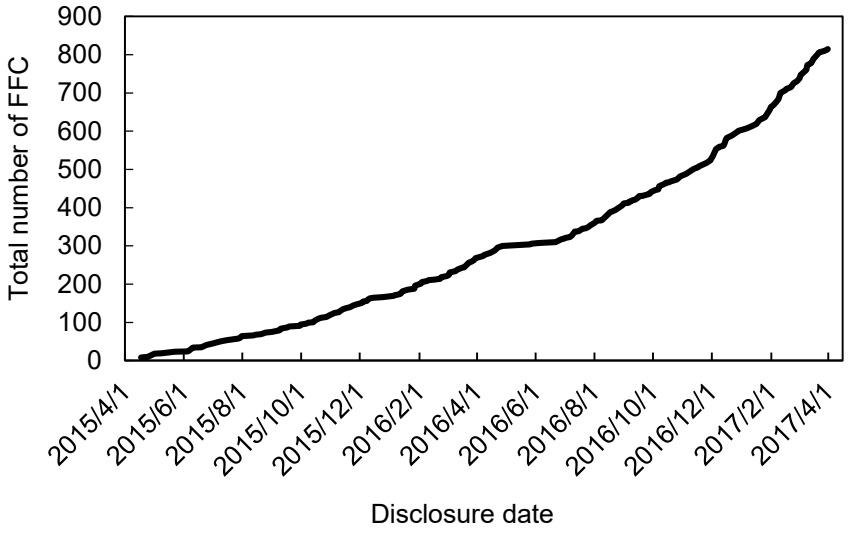

Figure B-1 The total number of FFC.
It was prepared with the information available on the CAA website as of March 31, 2017 (from notification number A1 to B513 excluding 8 withdrawn products). The total number of FFC was 815. FFC, Foods with Function Claims; CAA, Consumer Affairs Agency.

B.3 Food Business Operators

The food business operators of FFC were analyzed. As of March 31, 2017, the number of food business operators of FFC was 236 companies (excluding two withdrawn companies). By prefecture, Tokyo had the largest number of food business operators of FFC, followed by Osaka

and Fukuoka (Figure B-2). In addition, food business operators in 32 prefectures have submitted notifications, and the FFC system has been used by food business operators nationwide. Food business operators of FFC were categorized by capitalization (Table B-1). The result showed that even excluding food business operators with unknown capital, 58.9% of all had a capital of less than 300 million yen. In addition, 46.2% of all had a capital of less than 100 million yen. Thus, the FFC system has been widely used by small and medium-sized companies.

Table B-1 The number of food business operators of FFC by capital size.

Capital size	Number	%
≥ 500 million JPY	68	28.8
≥ 300 million JPY to < 500 million JPY	8	3.4
≥ 100 million JPY to < 300 million JPY	30	12.7
≥ 50 million JPY to < 100 million JPY	37	15.7
< 50 million JPY	72	30.5
Unknown	21	8.9

It was prepared with the information available on the CAA website as of March 31, 2017 (from notification number A1 to B513 excluding 8 withdrawn products). The information on capital size was obtained from the food business operator's website. The total number of food business operators is 236 (excluding 2 food business operators that have withdrawn FFC notification). FFC, Foods with Function Claims; JPY, Japanese yen; CAA, Consumer Affairs Agency.

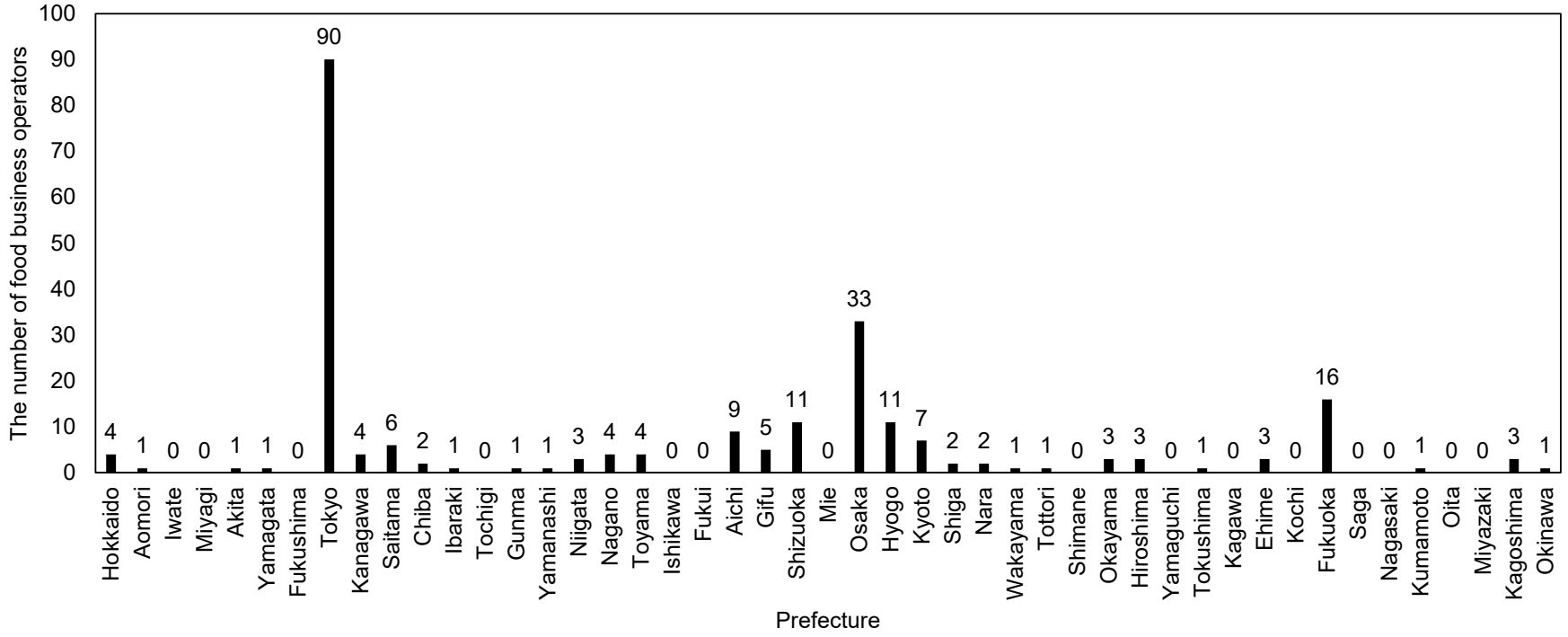

Figure B-2 The number of food business operators of FFC by prefecture.

It was prepared with the information available on the CAA website as of March 31, 2017 (from notification number A1 to B513 excluding 8 withdrawn products). The total number of food business operators was 236 (excluding 2 food business operators that have withdrawn FFC notification). FFC, Foods with Function Claims; CAA, Consumer Affairs Agency.

B.4 Food Categories

The food categories of FFC were analyzed. Of the total 815 products (excluding 8 withdrawn products), 358 (43.9%) were processed foods (supplement form), 451 (55.3%) were processed foods (others), and 6 (0.7%) were fresh foods (Table B-2). The reason for the small number of fresh foods in FFC could be that it is difficult to guarantee the content of functional substances. The quality of fresh foods is greatly affected by the place of origin, variety, climate, and so on. In addition, if there is a large difference in the quality of each piece, a full inspection may be necessary. Another reason could be that there is little scientific evidence for the physiological functions of fresh foods. These obstacles may have prevented fresh foods from entering the market for FFC. Therefore, support from the government and other experts is necessary. Based on these backgrounds, the Ministry of Agriculture, Forestry and Fisheries [4] and the National Agriculture and Food Research Organization [5,6] has released support materials regarding FFC of fresh foods.

Table B-2 The number of FFC by food types.

Type of food	Number	%
Processed food (supplement form)	358	43.9
Processed food (others)	451	55.4
Fresh food	6	0.7

It was prepared with the information available on the CAA website as of March 31, 2017 (from notification number A1 to B513 excluding 8 withdrawn products). The total number of FFC was 815. FFC, Foods with Function Claims; CAA, Consumer Affairs Agency.

B.5 Scientific Evidence

Under the FFC system, food business operators must submit scientific evidence for the proposed function claims either by the clinical trial of the finished product or by the systematic review (SR) of the finished product or its functional substance. It is also possible to submit a combination of these scientific pieces of evidence. Of the total 815 products (excluding 8 withdrawn products), 44 (5.4%) used the clinical trial of the finished product, 1 (0.1%) used the SR of the finished product, and 766 (94.0%) used the SR of the functional substance (Table B-3). Four products (0.5%) combined clinical trial of the finished product with SR of functional substance (Table B-3). One possible reason why many notifications used the SR on functional substance may be that almost the same SR can be used in various products, if there is a rational reason. This unique regulation of the FFC system contributes to reducing the cost and time burden of food business operators required to obtain scientific evidence.

Table B-3 The number of FFC by methods of evaluating function claims.

Methods of evaluating function claim	Number	%
A: Clinical trial of the finished product	44	5.4
B: Systematic review on the finished product	1	0.1
C: Systematic review on functional substance	766	94.0
D: Combination of A and C	4	0.5

It was prepared with the information available on the CAA website as of March 31, 2017 (from notification number A1 to B513 excluding 8 withdrawn products). The total number of FFC was 815. FFC, Foods with Function Claims; CAA, Consumer Affairs Agency.

B.6 Functional Substances

The functional substances contained a total of 815 FFC (excluding 8 withdrawn products) were listed along with the number and percentage of the products (Table B-4). The result showed that there were 83 functional substances (excluding one withdrawn). Because there were some FFC that contained multiple functional substances, the total number of functional substances did not match the number of FFC. Indigestible dextrin (114 products, 12.1%) had the largest number of FFC, followed by gamma-amino butyric acid (86 products, 9.1%) and docosahexaenoic acid (78 products, 8.3%). The functional substances selected by food business operators are probably influenced not only by marketability but also by the availability of scientific evidence and the processing characteristics. Based on these backgrounds, ingredients that have been widely used in FOSHU, such as non-digestible dextrin, gamma-amino-butyric acid, and bifidobacteria, as well as docosahexaenoic acid and eicosapentaenoic acid, which have many scientific evidence worldwide, could have been used in FFC.

Table B-4 The number of FFC by functional substances.

Functional substance	Number	%
Indigestable dextrin	114	12.1
Gamma-amino-butyric acid	86	9.1
Docosahexaenoic acid	78	8.3
Eicosapentaenoic acid	70	7.4
Sodium hyaluronate	51	5.4
Bifidobacterium	47	5.0
Pueraria thomsonii flower isoflavone	39	4.1
L-Theanine	39	4.1
Lutein	35	3.7
Glucosamine	28	3.0
Acetic acid	27	2.9
Soy isoflavone	23	2.4
Ginkgo flavone glycosides、terpene lactones	23	2.4
Undenatured type II collagen	19	2.0
Astaxanthin	15	1.6
Rice glucosylceramide	15	1.6
Zeaxanthin	14	1.5
Bilberry anthocyanin	14	1.5
Salacinol	12	1.3
Barley beta-glucan	12	1.3
Monoglucosyl hesperidin	12	1.3
Lactic acid bacteria	11	1.2
Reduced coenzyme Q10	11	1.2
Lycopene	10	1.1
Grabridin	9	1.0
Lactotripeptide	8	0.8
Methylated catechin	7	0.7
Beta-cryptoxanthin	7	0.7
Others	109	11.5

It was prepared with the information available on the CAA website as of March 31, 2017 (from notification number A1 to B513 excluding 8 withdrawn products). There were 83 functional substances listed on the CAA website (excluding 1 withdrawn substance). Although the total number of FFC was 815, the cumulative number of the functional substances was 945 because some of the FFC designated two or more functional substances. FFC, Foods with Function Claims; CAA, Consumer Affairs Agency.

B.7 Function Claims

Both the FOSHU and the FFC systems are the food labeling regulations for functional foods in Japan. In FFC, there are not only health claims in FOSHU, but also various other health claims. Then, the function claims of FFC were categorized. FFC could be categorized into 22 types, triglyceride, blood glucose level, intestine, visceral fat/body fat, skin, BP, eyes, joints, mental stress, cognitive function, sleep, bones, physical fatigue, cholesterol, body temperature, eye and nose discomfort, muscles, walking ability, blood lipid oxidation, liver, lower back, and fat metabolism (Table B-5). The most frequent function claims were triglycerides (161 products, 16.8%), followed by blood glucose level (103 products, 10.7%) and intestine (102 products, 10.6%). Function claims categorized as eyes, joints, mental stress, cognitive function, sleep, physical fatigue, body temperature, eye and nose discomfort, muscles, walking ability, blood lipid oxidation, liver, and lower back were not included in FOSHU. Thus, the FFC system has greatly expanded the area of health claims that can be labeled.

Table B-5 The number of FFC by category of function claim.

Category of function claim	Total number	%	Number of food type		
			Supplement form	Others	Fresh food
Triglyceride	161	16.8	31	130	0
Blood glucose level	103	10.7	19	84	0
Intestine	102	10.6	18	84	0
Visceral fat/Body fat	93	9.7	35	58	0
Skin	79	8.2	37	42	0
Blood pressure	75	7.8	20	55	0
Eyes	58	6.0	55	3	0
Joints	50	5.2	48	2	0
Mental stress	49	5.1	18	31	0
Cognitive function	39	4.1	29	10	0
Sleep	34	3.5	28	6	0
Bones	30	3.1	12	12	6
Physical fatigue	24	2.5	16	8	0
Cholesterol	22	2.3	5	17	0
Body temperature	14	1.5	1	13	0
Eye and nose discomfort	10	1.0	3	7	0
Muscles	6	0.6	4	2	0
Walking ability	5	0.5	4	1	0
Blood lipid oxidation	2	0.2	2	0	0
Liver	1	0.1	1	0	0
Lower back	1	0.1	1	0	0
Fat metabolism	1	0.1	1	0	0

It was prepared by the information available on the CAA website as of March 31, 2017 (from notification number A1 to B513 excluding 8 withdrawn products). Although the total number of FFC was 815, the cumulative number of the functional claims was 959 because some of FFC designated two or more function claims. FFC, Foods with Function Claims; CAA, Consumer Affairs Agency.

B.8 Perspectives

The number of FFC disclosed on the CAA website has been increasing progressively over time. Therefore, the market of FFC is expected to expand further. On the other hand, differentiation from competitors will be a challenge for food business operators. In the future, more food business operators may conduct clinical trials to develop new areas of health claims.

Under these circumstances, it is necessary to protect the results obtained through research and development as the rights of the inventor. The Japan Patent Office has revised its examination standards and began examining inventions for the use of food on April 1, 2016 [7]. Consequently, use inventions are now permitted for foods as well as for pharmaceuticals. Therefore, it may be important to develop patent strategies that consider not only claims for objects but also uses.

FFC are intended for people who do not suffer from diseases. Therefore, in principle, the evidence must be clinical trials conducted on individuals who do not have the disease. However, most of the clinical trials conducted in the world have been on diseased individuals. Some food business operators may have declined to submit notifications because they are unable to adopt previously reported evidence. This may be a particularly difficult problem for small and medium-sized companies and producers of fresh foods who are unable to conduct clinical trials themselves due to cost and technical reasons.

The FFC system accepts not only unpublished SRs but also published SRs as scientific evidence. Published SRs are more reliable in that they have been validated by experts in clinical trials and statistical analysis. However, published SRs have not been used much in the FFC system. One of the reasons for this could be the lack of published SRs of the functional

substances. Another possible reason could be that the SRs included people who are not eligible for the FFC system (e.g., diseased or underage people). For example, the SR, which verified how DHA affects the memory of healthy people, includes underage people [8]. Therefore, it would be difficult to apply it directly to the FFC system. If someone with expertise in clinical trials, statistical analysis, SRs, and meta-analysis is available, stratified analysis with appropriate exclusion of out-of-scope literature can be considered. However, this may be difficult for small and medium-sized companies and producers of fresh foods who have no experience in conducting clinical trials. Modification of the system to facilitate the use of existing evidence may encourage small and medium-sized companies and producers of fresh foods to develop FFC.

With the launch of the FFC system, food business operators than ever need to obtain appropriate scientific evidence to promote the health benefits of foods. It is hoped that consumers and food business operators will increasingly understand the FFC system and that this system will contribute to healthy life.

References

[1] Consumer Affairs Agency. What are "Foods with Function Claims"? 2015. https://www.caa.go.jp/policies/policy/food_labeling/information/pamphlets/pdf/151224_1.pdf. Accessed February15, 2021.

[2] Consumer Affairs Agency. The system of "Foods with Function Claims" has been launched! 2015. https://www.caa.go.jp/policies/policy/food_labeling/information/pamphlets/pdf/151224_1.pdf. Accessed February 15, 2021.

[3] Consumer Affairs Agency. The Foods with Function Claims database [in Japanese]. https://www.fld.caa.go.jp/caaks/cssc01/. Accessed February 15, 2021.

[4] Ministry of Agriculture, Forestry and Fisheries, Government of Japan. Technical Measures for Functional Labeling of Agricultural, Forestry and Fishery Products [in Japanese]. 2015. https://www.affrc.maff.go.jp/kinousei/gijyututekitaio.htm. Accessed February 15, 2021.

[5] National Agriculture and Food Research Organization. Project of functional agricultural products development [in Japanese]. https://www.naro.affrc.go.jp/project/f_foodpro/2016/063236.html. Accessed February 15, 2021.

[6] Maeda-Yamamoto M, Ohtani T. Development of functional agricultural products utilizing the new health claim labeling system in Japan. *Biosci Biotechnol Biochem*. 2018;82(4):554–563. doi:10.1080/09168451.2017.1422175

[7] Japan Patent Office. Revision of the Examination Guidelines for Patent and Utility Model. 2016. https://www.jpo.go.jp/e/system/trademark/gaiyo/bunrui/kokusai/h2803_kaitei.html. Accessed February 15, 2021.

[8] Yurko-Mauro K, Alexander DD, Van Elswyk ME. Docosahexaenoic acid and adult memory: a systematic review and meta-analysis. *PLoS One*. 2015;10(3):e0120391. doi:10.1371/journal.pone.0120391

List of Related Publications

Chapter 2

Naoki Yuda, Miyuki Tanaka, Akio Yamada, Daisuke Ochi, Koji Yamauchi, Fumiaki Abe, and Naoki Sakane. Antihypertensive effect of the casein-derived peptide Met-Lys-Pro in individuals with high-normal blood pressure or grade 1 hypertension –A randomized, double-blind, placebo-controlled, parallel-group trial–. *Japanese Pharmacology Therapeutics*. 2018;46(4):529–537.

Chapter 3

Naoki Yuda, Miyuki Tanaka, Koji Yamauchi, Fumiaki Abe, Izumi Kakiuchi, Kyoko Kiyosawa, Mitsunaga Miyasaka, Naoki Sakane, and Masahiko Nakamura. Effect of the casein-derived peptide Met-Lys-Pro on cognitive function in community-dwelling adults without dementia: a randomized, double-blind, placebo-controlled trial. *Clinical Interventions in Aging*. 2020;15:743–754.
doi:10.2147/CIA.S253116

Chapter 4

Naoki Yuda, Miyuki Tanaka, Masahiko Tokushima, and Fumiaki Abe. Safety evaluation of high-dose intake of casein-derived peptide Met-Lys-Pro in healthy adults: a randomized, double-blind, placebo-controlled trial. *Food Science & Nutrition*, 2021;9(2), 662–671. doi:10.1002/fsn3.2028

Appendix A

Naoki Yuda, Miyuki Tanaka, Manabu Suzuki, Yuzo Asano, Hiroshi Ochi, and Keiji Iwatsuki. Polyphenols extracted from black tea (*Camellia sinensis*) residue by hot-compressed water and their inhibitory effect on pancreatic lipase *in vitro*. *Journal of Food Science*. 2012;77(12):H254–H261.
doi:10.1111/j.1750-3841.2012.02967.x

Appendix B

Naoki Yuda. Understanding Foods with Function Claims through analytical results of notification status [in Japanese]. *Journal of Nutritional Food*. 2017;16(1):1–10. doi:10.20618/jhnfa.16.1_1