Review

Japanese Perspective and Prospect Beyond the Pandemic of the Asian Society of Clinical Pathology and Laboratory Medicine

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ABSTRACT

The Asian Society for Clinical Pathology and Laboratory Medicine (ASCPaLM) is a non-profit organization that was founded in 1975 for the societies primarily engaged in laboratory medicine (clinical pathology). Its aim is to enhance the development and practice of laboratory medicine to promote better support the health and well-being of the communities it serves. The organization support its constituent societies in Asia, as well as its other members through communication, cooperation in education, research and practice. The Japanese Society for Laboratory Medicine (JSLM) has played a crucial role in establishing ASCPaLM and continues to be actively involved in its operation. It needs to keep to demonstrate leadership in these activities, leveraging its innovation initiative, particularly through collaboration with industry sectors. The ASCPaLM contributed to combatting the COVID-19 pandemic by sharing experiences and knowledge, with clinical pathologists and laboratory professionals at the forefront of laboratory practice and services. To address the weakness of communities that the pandemic has exposed, it is an important time to take action and achieve the the Sustainable Development Goals (SDGs), by promoting innovation, technology transfer, and continuous quality improvement of the laboratory practice to meet requirements in international standards.

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Key Words
Asia, clinical pathology, laboratory medicine, congress, organization, COVID-19

I. Aim and history of the organization

1. Aim

The Asian Society for Clinical Pathology and Laboratory Medicine (ASCPaLM) is a non-profit organization that was founded in 1975 for the societies primarily involved in laboratory medicine (clinical pathology)[1]. The organization's aim is to improve the development and practices of laboratory medicine to promote the health and well-being of the communities it serves (**Table 1**). The ASCPaLM strives to support its constituent societies in Asia and other members globally by facilitating communication, cooperation in education, research and practice. Its goal is to promote the information exchange on activities, experiences, and knowledge among Asian countries and contribute to health, medical care, and welfare both in and outside the region by maintaining high-quality of clinical laboratory practice.

2. History

The ASCPaLM has a long history dating back to the early days of laboratory medicine, and has successfully facilitated collaboration among the clinical laboratory societies throughout Asia. The history of ASCPaLM dates back to the Korean-Japanese Conference of Clinical Pathology, which held the first conference in Seoul, South Korea in 1975. Since then it has been held every two years, alternating between Japan and South Korea. In time, other Asian countries including Taiwan joined as full-members. At the 9th conference in 1990, it was reorganized and renamed as 'the Asian Conference of Clinical Pathology: ACCP'. The 1st ACCP was held in Sapporo, Japan, under the co-chairmanship of Profes-

Table 1-1 Organization & Committees

1. Organization and its Name	The organization is a "non-profit" organization for the societies whose main activities are in laboratory medicine (clinical pathology). It shall be known as "the Asian Society for Clinical Pathology and Laboratory Medicine". Abbreviation of the Society shall be ASCPaLM.
2. Purpose	The purpose of the Asian Society for Clinical Pathology and Laboratory Medicine herein after referred to as the Society exists 1) to promote improvement in the health and well-being of the communities it serves through improving the development and practice of laboratory medicine (clinical pathology) and 2) to support not only its constituent societies in Asia but also its other members through communication cooperation in education research and practice.
3. Membership	3.1 Geographic eligibility for membership shall apply to societies and normally having its activities in countries/areas in Asia and the areas shall be based on geography and not political considerations. 3.2 Classes of memberships are Full membership (Constituent Society), Affiliate membership, and Corporate membership. 3.2.1 Full Membership (Constituent Society): Full membership is available to the recognized and established national/area societies of laboratory medicine (clinical pathology) operated mainly by physicians in the area and has the voting right and the eligibility for election. 3.2.2 Affiliate membership has three categories 1) A society from a country/area within Asia may apply to be an Affiliate membership if there is no recognized and established society of laboratory medicine (clinical pathology) in that country/area. 2) An international or national society not from Asia but having an interest in laboratory medicine (clinical pathology) may apply for an Affiliate membership. 3) An individual physician from a country/area in Asia may apply to be an Affiliate membership for a certain period of time if there is no recognized and established society of laboratory medicine (clinical pathology) or no appropriate affiliate membership society in that country/area and shall be encouraged to organize a national/area society of laboratory medicine. 3.2.3 Corporate membership: Corporate membership is open to manufacturers and also distributors of clinical laboratory equipment and reagents. 3.2.4 Each Affiliate and Corporate member may send one observer to the meetings of the Society.The observers will not have the voting rights and the eligibility for election. 3.2.5 There shall be only one class of membership for any one geographical area except for Corporate membership.
4. Council	4.1 The affairs of the Society shall be conducted by a Council. 4.2 Each constituent society shall appoint two delegates to Council who shall be a member of the governing body of that constituent society. The society may appoint a deputy who may attend a Council Meeting in place of the delegate. 4.3 Members of the Executive Committee shall be members of the Council ex-officio. 4.4 Affiliate and Corporate members shall not be a member of Council. 4.5 At least a half (50%) plus one of the total memberships of the Society present at the Council Meeting shall form a quorum. 4.6 The Executive Committee may determine matters by postal or e-mail ballot of the Executive Committee or by postal or e-mail ballot of the members of Council as it shall deem fit. At least one half of either the Executive Committee or Council members' replies must be received before such matters are considered valid. 4.7 Important issues such as proposal for changes of bylaws should be decided by more than a half of attending council members or members replying the E-mail. 4.8 Council shall meet every year in which one is at times and in places to coincide with the Congress which is scheduled to be held every two years or on occasions as deemed necessary by the Executive Committee. 4.9 At least three months' notice will be given of a Council Meeting and particulars of its agenda will be circulated to all members at least one month in advance of the meeting. 4.10 The following points will be considered at the Council Meeting: 4.10.1 The previous term's accounts and report of the Executive Committee. 4.10.2 The election of office-bearers for the next term of office. 4.11 The admission of new members to the Society shall be decided by Council by a simple majority vote.

Table 1-2 Organization & Committees

5. Executive Committee	5.1 There shall be an Executive Committee which shall be responsible for the day-to-day affairs of the Society. 5.2 The Executive Committee shall consist of the President, the President-Elect (the Vice- President), Past-President, Secretary, Treasurer, and Auditors. 5.3 The term of office of the President, the Vice President. Past-President, Auditors, and Treasurer shall be two years whichever is the longer. The President the Vice-President Past-President Auditors and Treasurer shall retire on completion of the term of office and shall be ineligible for re-election for a further term in the same office. 5.4 The first term for the Secretary shall be three years. After the first term it shall be two years or from one Council Meeting to the next Council Meeting whichever is the longer. The Secretary shall be eligible for re-election. 5.5 The duties of the office-bearers are as follows: 5.5.1 The President shall act as Chairman at all Council and Executive Committee Meetings. He or she or a designate shall represent the Society in its dealings with outside organizations. The President or designate will be a member ex-officio of all Standing Committees of the Society. 5.5.2 The Vice-President shall deputize for the President in the latter' s absence. 5.5.3 The Secretary shall keep all records except financial of the Society and shall be responsible for their correctness. He or she will keep Minutes of all Council and Executive Committee Meetings. 5.5.4 The Treasurer shall keep an account of all monies collected and disbursed on behalf of the society and all other monetary transactions and shall be responsible for their correctness. All monies will be deposited in a bank in a designated country to be named by the Committee.
6. Funding of the Society	6.1 With the advice from Council, the Executive committee shall set the membership fee (annual due). 6.2 There shall be no membership fee for Affiliate and Corporate membership.
7. Amendment of the Constitution	7.1 The Constitution will be amended by an affirmative vote of two thirds of the membership of the Council. 7.2 Notice of proposed amendments of the Constitution shall be given by the Secretary in writing no less than three months prior to a vote to amend the Constitution.
8. Audit	Auditors will be required to audit the accounts of the Society and present a report upon them at the Council Meeting. They may be required by the President to audit the Society' s accounts for any period within their tenure of office and make a report to the Council.

sor Teiichi Sasaki and Professor Ichiro Kurokawa from Sapporo Medical College (**Table 2**). The ACCP continued to be held every two years until the 8th meeting in 2004, when the name was changed to the current ' Asian Society of Clinical Pathology and Laboratory Medicine, ASCPaLM[1]. Today, the ASCPaLM has five full-member countries: i.e., Japanese Society of Laboratory Medicine (JSLM), Korean Society for Laboratory Medicine (KSLM), Indonesian Association of Clinical Pathologists (IACP), Taiwan Society of Clinical Pathologists (TSCP) and Mongolian Association of Laboratory Medicine (MALM). All of representatives and congress are endorsed by their respective national society of laboratory medicine.

II. Japan's efforts

1. Host of the Congresses

In Japan, following the 1st ACCP in 1990, the 5th ACCP was held in Kochi in 1998 under the chairman of Professor Jiro Endo from Shimane Medical University. The 9th ASCPaLM congress was held in Kobe in 2006 under the chairman of Professor Shunichi Kumagai from Kobe University, and the 12th was held in Kyoto in 2012 under the chairman of Professor Hakuo Takahashi from Kansai Medical University, and the 17th was in Kobe in 2022 under the chairman of Professor Hayato Miyachi from Tokai University and Nitobe-Bunka College[2].

2. Financial and academic support

To promote cooperation with international organizations in clinical pathology and laboratory medicine, JSLM has closely worked with international organizations like ASCPaLM and WASPaLM[3][4]. In 1989, JSLM established the Asian Exchange Fund to provide financial and academic support to ASCPaLM congress held in Asian member countries. To encourage young scientists, in 2009, JSLM created an award to recognize and support their research presentation at international conferences, such as the Congresses of the ASCPaLM and the World Congresses of WASPaLM.

3. Collaboration with WASCPaLM

Collaboration between ASCPaLM and WASCPaLM has been actively developed and promoted by the Japanese Presidents of WASPaLM[5][6]. In 1992, at the 2nd ACCP Conference, a WASPaLM Bureau Meeting was held concurrently with the suggestion of Professor Tadashi Kawai from Jichii University, as the President of WASPaLM.

Table 2 Congress of the Asian Society of Clinical Pathology and Laboratory Medicine

Congress	Year	Chairman	Venue
1 ACCP	1990	Teiichi Sasaki and Ichiro Kurokawa	Sapporo, Japan
2	1992	Jay Sik Kim	Cheju, South Korea
3	1994	Jui-San Chen	Taipei, Taiwan
4	1996	Rustadi Sosrosumihardjo	Yogyakarta, Japan
5	1998	Jiro Endo	Kochi, Japan
6	2000	Soon Ho Kim	Busan, South Korea
7	2002	Jang Hwa Lee	Kahsung, Taiwan
8 ASCPaLM	2004	A.K. Amaan	Medan, Indonesia
9	2006	Shunichi Kumagai	Kobe, Japan
10	2008	Namid Munkhtuvshin	Ulaanbaatar, Mongolia
11	2010	Hardjieno	Jakarta, Indonesia
12	2012	Hakuo Takahashi	Kyoto, Japan
13	2014	Oh Hum Kwon	Seoul, South Korea
14	2016	Tjin-Shing Jap	Taipei, Taiwan
15	2018	M.Oyundelger	Ulaanbaatar, Mongolia
16	2021	Aryati MS	Virtual, Indonesia
17	2022	Hayato Miyachi	Kobe, Japan
18	2024	Gye-Cheol Kwon	Seoul, South Korea

ACCP, Asian Conference of Clinical Pathology; ASCPaLM, Asian Society for Clinical Pathology and Laboratory Medicine

This promoted exchange between ACCP and WASPaLM, leading to approve of the opening of WASPaLM's administrative office in Japan.

Under the leadership and guidance of Professor Masami Murakami from Gunma University, as the President of WASPaLM, a joint symposium between ASCPaLM and WASPaLM was held during the World Congress of WASPaLM held in Kyoto, Japan in 2017, as well in Xi'an, China in 2019. In 2022, JSLM session as an ASCPaLM member was held during the World Congress of WASPaLM at Punta del Este, Uruguay, which was postponed for one year due to the coronavirus pandemic.

III. Combatting COVID-19 pandemic

1. Clinical pathologists at the forefront of combatting COVID-19

In 2020, as the President of ASCPaLM, Professor Hayato Miyachi from Tokai University announced to share activities, experiences and knowledges not only among our societies of ASCPaLM and their communities, but also societies and communities from other regions in the world, as follows. The COVID-19 pandemic was an unprecedented experience in human history7). It brought to light the crucial role of laboratory services in combatting the spread of the disease. Clinical pathologists and laboratory professionals were at the forefront of the medical laboratories and thus patient care in the medical facilities. All clinical pathologists in each member country of ASCPaLM made the best effort in the laboratory practice for the care of patients and general population by managing laboratory practices and services. Some of member countries of ASCPaLM successfully controlled the spread of COVID-19 earlier than other areas of the world, by taking an advantage of the laboratory tests for corona virus. The experience and knowledge gained through the laboratory practice in Asia would be useful and helpful for controlling the outbreak in the world and also for preparing for the future surges.

2. The virtual Congress at the pandemic

The 16th ASCPaLM Congress was held in Indonesia virtually for the first time in the history of ASCPaLM [8]. Originally scheduled in September 2020, the meeting was postponed to until January 2021, due to pandemic situation. While clinical pathologist played a pivotal role in COVID-19 pandemic, they must face complex challenges collectively. Therefore, the main theme of Congress was “Unite for Combatting COVID-19”. The goal of Congress was to share knowledge and experience among country members of the society about COVID-19 situation, issues, strategies, and challenges, as well as education of clinical pathologists in pandemic situation. Two symposiums were held under the main theme in the congress: Symposium I focused on the roles of clinical pathologists in the COVID-19 pandemic and Symposium II focused on adaptations for residential programs of clinical pathologists during the COVID-19 pandemic.

3. The Congress toward the post-corona era

The 17th ASCPaLM Congress took place in October 2022 in Kobe, making a historic moment in the post-corona era for international exchanges [2]. The main theme

of the congress was the Sustainable Development Goals (SDGs), which aimed to address the weakness of communities that the pandemic has exposed. This was an important time to take action and achieve the SDGs in the community that laboratory medicine (clinical pathology) serves, by promoting the innovation, technology transfer, and continuous quality improvement of the laboratory practice to meet requirements in international standards. Laboratory accreditation under the international standard is important as an approach for progress in the goal 3 of SDGs which aspires to ensure healthy lives and promote well-being for all at all ages. Collaboration and partnership are critical in these efforts. Two symposiums were held under the main theme of SDGs in the congress: Symposium I focused on the impacts of quality laboratory practice on SDGs, and Symposium II focused on preparedness for the next-pandemic.

IV. Perspective and prospects beyond the pandemic

1. Personnel development initiative

Medical laboratories play a crucial role in modern medical practices, as laboratory reported results are integral to physicians' decision-making processes. They help identify risk factors, screen and detect predispositions to symptomatic diseases, confirm or negate diagnoses, guide patient management, and individually tailor therapy for personalized medicine. Clinical pathologists are expected to take a role in knowledge-integrated services including comprehensive approaches in the diagnosis of diseases and consultation, customer intimacy, leadership in multi-disciplinary team-based medicine, which is laboratory-based and the patient centered[9]. In order to support the health and well-being of the communities in modern medical practices, it is more important than ever to invest in human resource development in laboratory medicine and take an initiative in an international community.

In 2017, Japan, South Korea, Taiwan, Indonesia and Mongolia signed a memorandum of understanding (MOU) to promote mutual exchange in the fields of clinical pathology and laboratory medicine. MOU aimed facilitate cooperation in education, standardization, and academic presentations. As a result, coordination is currently being underway with Asian countries to share educational classes for clinical pathology residents.

2. Quality laboratory practice to meet requirements of the international standards

Compliance with international standards such as ISO 15189, which is the requirements for quality and competence of medical laboratories, demonstrates its reliability and objectivity for clinical laboratory, emphasizing an importance of the laboratory accreditation[10]. There is consistent evidence that shows that accreditation programs improve the process of care provided by healthcare services including clinical trials. The 17th ASCPaLM Congress addressed the importance of quality assurance of medical laboratory service through accreditation under the international standard. The congress also introduced ATLAS (Asian clinical TriaLs network for cAncerS) project, which was started by National Cancer Center Hospital (NCCH) in 2020, with support by Japan Agency for Medical Research and Development (AMED) [11]. The project aimed to develop a robust Asian Clinical Trials Network, with existing partnerships reinforced in South Korea, Taiwan, and Singapore, and the clinical trial network expanded to the Association of Southeast Asian Nations (ASEAN) such as Thailand, Malaysia, the Philippines, and Vietnam. The NCCH is sharing its budget with Asian sites to reinforce their clinical trial infrastructure, offering capacity building programs on clinical trials and high-quality genomic profiling test, and proposing several international clinical research. The project includes a system for collecting samples from Asian countries, central pathological review, genomic profiling tests after specimen quality control, and reporting results to the facilities in Asia. Monitoring the quality of specimen and providing feedback to the site for improvement was also implemented. In the era of genomic medicine, it is more important than ever for medical laboratory to be managed by competent personnel and to comply with international standards such as ISO 15189[12]. Accreditation of medical laboratories that perform genomic profiling tests under ISO 15189 in Asia has been supported by Japan, by implementing an educational course.

3. Innovation initiative

Asia is a region that has experienced remarkable economic development, and with this growth, the field of clinical laboratory medicine is also expanding in each country. As a result, the number of participants in academic meetings has increased as well. For example, in 2016, the largest delegate ever in the history with a total of 88 clinical pathologists participated in the 14th ASCPaLM Congress held in Taipei, Taiwan. In Indonesia, the speciality of clinical pathology is ranked highest among medical doctors, followed by surgery, and then internal medicine and other specialities. This reflects the government's recognition and support of laboratory tests when assessed by a clinical pathologist. In addition to five full-membership countries, each congress includes an increasing number of participants from other Asian coun-

tries such as China, Thailand, the Philippines, Pakistan, Malaysia, Singapore, Nepal, and India. Efforts have been made to increase the number of the Board and Council members by recruiting member countries among Asian participants.

Collaboration with Asian countries is of great significance for the Japanese industry. Due to the declining birthrate and aging population, Japan is experiencing a decline in its working population. The medical industry is expected to improve its international competitiveness as a remaining growth industry in Japan. Many Japanese manufacturers for in vitro diagnostics are aiming to expand their markets overseas, especially in Asia.

At the 51st Annual Meeting of the Japanese Society of Clinical Laboratory Automation (JSCLA) held in Yokohama, Japan, in October 2019, ASCPaLM collaborated with the Japanese Association of Clinical Laboratory Systems (JACLaS) which supports member companies in promoting their business domestically and overseas [13]. To this end, the Asian session with JACLaS was held, and the general exhibition JACLaS EXPO was internationalized for users from Asian countries. At the 17th ASCPaLM Congress held in Kobe, Japan, in 2022, JACLaS had co-sponsored sessions and provided a travel award for foreign scientist presenters. JSLM needs to keep to demonstrate leadership in the activities, leveraging its innovation initiative, particularly through collaboration with industry sectors.

Acknowledgements

I would like to express my gratitude to all the members of the Asian Society for Clinical Pathology and Laboratory Medicine for their dedication to fulfilling its mission and responsibilities, especially the members of the executive committee who oversee its day-to-day operations.

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Editorial

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Construction of new formula for estimating corrected Ca concentration the modified bromocresol purple method

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In this issue of Laboratory Medicine International, Ishigaki et al.' s paper : "A new equation for estimating iCa over a wide range of serum albumin concentrations measured by the modified bromocresol purple method", has been published. Construction of a new formula for estimating the corrected Ca (cCa) concentration using a modified bromocresol purple (BCP) method is an interesting and clinically important development.

Ionized Ca (iCa) is 50% of the total serum, 40% exists as protein-bound forms, mainly albumin (Alb), and 10% exists as chemically bound forms[1]. Therefore, serum Ca concentration measurements must be corrected by the serum Alb concentration.

Since the amount of protein-bound Ca decreases in hypoalbuminemia, the serum Ca concentration may not reflect the actual Ca concentration. For this reason, Payne' s formula (cCa (mg/dL) =tCa (mg/dL) +4–Alb (g/dL)) is most commonly used in patients with hypoalbuminemia in Japan[2-4]. However, because the cCa concentration is only an estimated value of the Ca concentration, it does not reflect the true Ca concentration. A characteristic of iCa is that as the pH decreases, the number of Alb-binding sites in iCa decreases and the ionization rate of Ca in serum increases[5].

Ishigaki et al. point out three problems with Paine' s formula: 1) the bromocresol green (BCG) method has cross-reactivity with proteins other than Alb (globulin, C-reactive protein, etc.), 2) iCa is not considered an index , and 3) it is applicable only when Alb concentrations are low.

In this study, a new formula was constructed to estimate the cCa concentration over a wide range of serum Alb concentrations using a modified BCP method corrected to pH 7.4 (cCa (mg/dL) =tCa (mg/ dL) +0.4 (4.6–Alb (g/dL)).

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Editorial

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Genetic polymorphisms associated with alcohol dependence in a Japanese archipelago population.

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The paper is by Mikami et al., entitled: "Association between delta opioid receptor gene polymorphisms and alcohol dependence in a Japanese archipelago population". The genetic relevance of alcohol dependence in a Japanese archipelago population is examined in terms of genetic polymorphisms of delta opioid receptors associated with alcohol-mediated processes in the brain.

It is clear that genes are involved in causing alcohol dependence. It is known that Single Nucleotide Polymorphisms (SNPs) of alcohol dehydrogenase 1B (*ADH1B*) and aldehyde dehydrogenase 2 (*ALDH2*), which are involved in alcohol metabolism, markedly influence the susceptibility to alcohol dependence. A number of other genes have been reported to be involved in alcohol dependence, including a polymorphism (rs6265) in the brain-derived neurotrophic factor (*BDNF*) gene.

It was suggested that *OPRD1* gene polymorphisms (rs 678849 and rs 2234918) may not be associated with alcohol dependence in the Japanese archipelago population. However, since different results may be obtained by increasing the size of the sample, we look forward to future studies on this.

The elucidation of genetic associations in alcohol dependence may lead to the identification of new therapeutic targets and development of therapeutic agents.

Original

New formulas for iCa estimation over a wide range of serum albumin concentrations measured by a modified bromocresol purple method

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ABSTRACT

Although the serum calcium concentration is affected by the serum albumin concentration and requires use of a formula, previous formulas were constructed using the bromocresol green (BCG) method, which has problems with accuracy. In this study, we constructed a new calcium formula using a modified bromocresol purple method, which overcame the limitations of the BCG method. The new formulas were constructed with the pH, ionized calcium (iCa), calcium, and albumin values of 706 patients. Sensitivity, specificity, and weighted kappa coefficient were evaluated with the values of 50 hemodialysis patients. We developed three formulas: to estimate iCa directly (Formula 1), to estimate iCa corrected to pH 7.4 (Formula 2), and to evaluate iCa corrected to pH 7.4 as an index (Formula 3). Using hemodialysis patients for validation, Pre-correction calcium and Formula 1 were tended to be classified as hypocalcemia than iCa or iCa corrected to pH 7.4, while Payne's formula and Kidney Disease Outcomes Quality Initiative's formula 2 were tended to be classified as hypercalcemia. Based on the weighted kappa coefficient, Formula 3 as corrected calcium was the best for assessment of calcium conditions. Since the serum calcium is widely used in daily practice, Formula 3 may be the most useful.

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Key Words
Bromocresol green method, Calcium formula, Ionized calcium, K/DOQI, Payne's formula, Modified bromocresol purple method

I. Introduction

Measurement of ionized calcium (iCa) is important for evaluating a patient's calcium level because of its direct role in physiological functions such as nerve and muscle excitation, blood coagulation, cell membrane permeability, and enzyme activation. However, because the measurement of iCa requires strict handling of the sample from blood collection to analysis [1], its determination in daily practice is limited. Instead of measurement iCa, corrected Ca (cCa), which is the total serum Ca (tCa) corrected by the albumin (Alb) concentration, is widely used [2)3)].

On the other hand, there are three problems with these formulas. First, these formulas use the Alb concentration measured by the bromocresol green (BCG) method,

which has been reported to react with other proteins [4)5)]. Second, these formulas do not take into account iCa as an index. Some studies comparing iCa and cCa have reported that cCa is a poor predictor of iCa abnormalities compared to uncorrected tCa [6)7)]. And finally, these formulas are only used for low Alb concentration.

The aim of this study was to construct new formulas to estimate or evaluate iCa for all Alb concentration based on the Alb measured by a modified bromocresol purple (mBCP) method which overcome the limitations of the BCG method [8)]. The mBCP method has been reported to show a strong correlation with immunonephelometry as the gold standard than with the BCG method [9)10)].

Because the iCa concentration fluctuates with pH, we first have constructed a formula to estimate iCa using pH, tCa, and Alb as variables. Then, we have also generated a formula to predict iCa corrected to pH 7.4 so that iCa can be easily estimated using only the Alb concentration. And finally, because tCa is widely used in daily practice, we have constructed a new formula for cCa that can be used to evaluate iCa more accurately than previous formulas [2)3)].

II. Materials and methods

1. Patients

To establish the new Ca formulas, we compiled the pH, iCa, tCa, and Alb values of 706 patients 20 years old and above whose arterial or venous blood gas analysis and biochemical tests were ordered and measured at the same time between January 2014 and October 2017 at Kyushu University Hospital and whose samples had pH values of 7.20–7.60.

Next, to test the usefulness of the newly constructed Ca formulas, we obtained the values of 50 hemodialysis patients who had agreed with written informed consent at Kyushu University Hospital before hemodialysis (**Table 1**). This study was approved by the Kyushu University Institutional Review Board for Clinical Research (approval number: 30-394, 2022-7). Since the concentration of iCa changes depending on the volume of blood collected and the time from blood collection to measurement [1)11)], we collected the required volume of blood and measured the iCa concentration within 15 min of blood collection. For iCa and pH, blood was collected using a BD Preset Syringe with Heparin for Arterial Blood (Becton, Dickinson and Company, Franklin Lakes, New Jersey). For tCa and Alb, blood was collected using a Insepack II-D tube (Tokuyama Sekisui Co., Ltd., Yamaguchi, Japan), then it was coagulated and centrifuged, and the serum was used for the measurement.

We examined the sensitivity, specificity, and weighted kappa coefficient of each formula (**Figure 1**). Pre-correction Ca, Payne's formula [2)], Kidney Disease Outcomes Quality Initiative's (K/DOQI's) formula 1 and K/DOQI's formula 2 were used for comparison [3)].

2. New formula

A formula for direct estimation of iCa was constructed by multiple regression analysis with pH, tCa, and Alb as variables (Formula 1). Because iCa and pH show a linear relationship from pH 7.2 to pH 7.6 [12)], a formula to estimate iCa corrected to pH 7.4 was constructed by multiple regression analysis with tCa and Alb (Formula 2). On the other hand, because tCa is frequently used in daily practice instead of iCa, cCa formula to evaluate iCa corrected to pH 7.4 as an index was constructed with tCa and Alb. To minimize the effect of a correction factor at normal levels of Alb, the reference interval of Alb was included in the new formula, and the value of X that was most closely correlated with iCa corrected to pH 7.4 was

Table 1 Patient demographics and laboratory values

Variable	Data for construction of new calcium correction formulas	Hemodialysis patients
Total	706	50
Age (year; mean ± SD)	58.9 ± 18.1	62.2 ± 11.5
Male (%)	411 (58)	29 (58)
Serum chemistries		
Total albumin (g/dL; mean ± SD)	3.16 ± 0.81	3.19 ± 0.62
Total calcium (mg/dL; mean ± SD)	8.61 ± 0.96	8.55 ± 0.59
Blood gus tests		
pH (mean ± SD)	7.392 ± 0.062	7.360 ± 0.039
iCa corrected to pH 7.4 (mmol/L; mean ± SD)	1.117 ± 0.113	1.155 ± 0.055
iCa (mmol/L; mean ± SD)	1.113 ± 0.116	1.130 ± 0.057

Total albumin (g/L) = 10 × Total albumin (g/dL), Total calcium (mmol/L) = 0.2495 × Total calcium (mg/dL)

iCa corrected to pH 7.4 was calculated with pH show a linear relationshio
from pH 7.2 to pH 7.6. iCa corrected to pH 7.4=iCa[1+0.53(pH–7.4)]

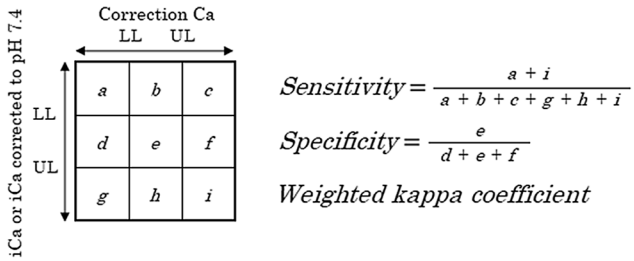

Figure 1 Method for evaluating the sensitivity, specificity, and weighted kappa coeffi-cient. LL: lower limit, UL: upper limit. Sensitivity: percentage of patients de-termined to be hypercalcemic or hypocalcemic using iCa or iCa corrected to pH 7.4. Specificity: percentage of patients determined to be within the refer-ence range using iCa or iCa corrected to pH 7.4. Weighted kappa coefficient: used to evaluate the agreement with iCa or iCa corrected to pH 7.4 on an or-dinal scale. Even if the determination is discrepant, the agreement score will be higher for *b*, *d*, *f* , and h than for *c* and *g*.

determined by Pearson's correlation coefficient (Formula 3).

Formula 1: Direct estimation for iCa with pH, tCa, and Alb as variables

Formula 2: Direct estimation for iCa corrected to pH 7.4 with tCa and Alb as variables

Formula 3: cCa=tCa+X([the median value of the reference interval of Alb]–Alb)

R version 4.2.0 was used for weighted kappa coefficient and multiple regression analysis.

3. Laboratory Tests

iCa and pH were measured using a Rapidpoint 500 system (Siemens Healthineers AG, Erlangen, Bayern). An enzymatic method, Accuras Auto Ca (Shino-Test Corporation, Tokyo, Japan), was used for the tCa measurement, and the Aqua-auto Kainos ALB test Kit (KAINOS Laboratories, Inc. Tokyo, Japan), an mBCP method, was used for the Alb measurement. The LABOSPECT 008 (Hitachi High-Tech Corporation, Tokyo, Japan) was used for the biochemical analysis. The reference intervals for Alb and tCa were 4.1 g/dL–5.1 g/dL and 8.6 mg/dL–10.1 mg/dL, respectively [13]. The iCa reference interval ranged from 1.13 mmol/L–1.33 mmol/L and was preset by the blood gas analyzer. The reference interval for iCa corrected to pH 7.4, 1.12 mmol/L–1.24 mmol/L, was obtained from 80 healthy volunteers.

III. Results

To estimate iCa directly, a multiple regression analysis was performed using pH, tCa, and Alb, and the new formula estimated iCa (eiCa)=0.113tCa–0.043Alb–0.338 pH+2.781(Formula 1) was obtained. The p-value for all variables was $p<0.01$ and the adjusted R-squared value was 0.71. To estimate iCa corrected to pH 7.4, multiple regression analysis was performed using tCa and Alb, and the formula eiCa(pH7.4)=0.116tCa–0.045Alb+0.260(-Formula 2) was generated. The p-value for all variables was $p<0.01$, and the adjusted R-squared value was 0.71. For Formula 3, the median value of the reference interval of Alb was 4.6 g/dL since the reference interval for Alb is 4.1–5.1 g/dL [13]. To determine the value of X that was most closely correlated with iCa corrected to pH 7.4, the Pearson's correlation coefficient was checked at intervals of 0.1 from 0.0 to 1.0, and the value of X was determined to be 0.4 ($r=0.843$). Thus, Formula 3 was cCa=tCa+0.4(4.6–Alb).

We evaluated the agreement between each formula and the reference interval for iCa or iCa corrected to pH 7.4 using the data from 50 dialysis patients (**Figure 2**). Pre-correction Ca and Formula 1 were tended to be classified as hypocalcemia than iCa or iCa corrected to pH 7.4, while Payne's formula and K/DOQI's formula 2 were tended to be classified as hypercalcemia. Formula 1 had the highest sensitivity at 100%, followed by Formula 2 (90.5%), and Pre-correction Ca (76.2%). Formula 3 had the highest specificity at 93.1%, followed by K/DOQI's formula 2 (89.7%), and Payne's formula (79.3%). Using the weighted kappa coefficient, Formula 3 (0.68) performed the best, followed by Formula 2 (0.64) and Formula 1 (0.54) (**Table 2**).

IV. Discussion

To evaluate blood Ca levels in accordance with a patient's pathological state, it is necessary to measure iCa, which indicates the calcium bioactivity. However, the measurement of iCa requires strict preanalytical conditions and specialized equipment [1], and thus tCa is usually measured instead. Since the concentration of tCa is affected by the Alb concentration, Ca formulas have been reported by Payne et al. [2], as well as others [3][6][7] that take into account the Alb concentration. On the other hand, the BCG method has been reported to react with other

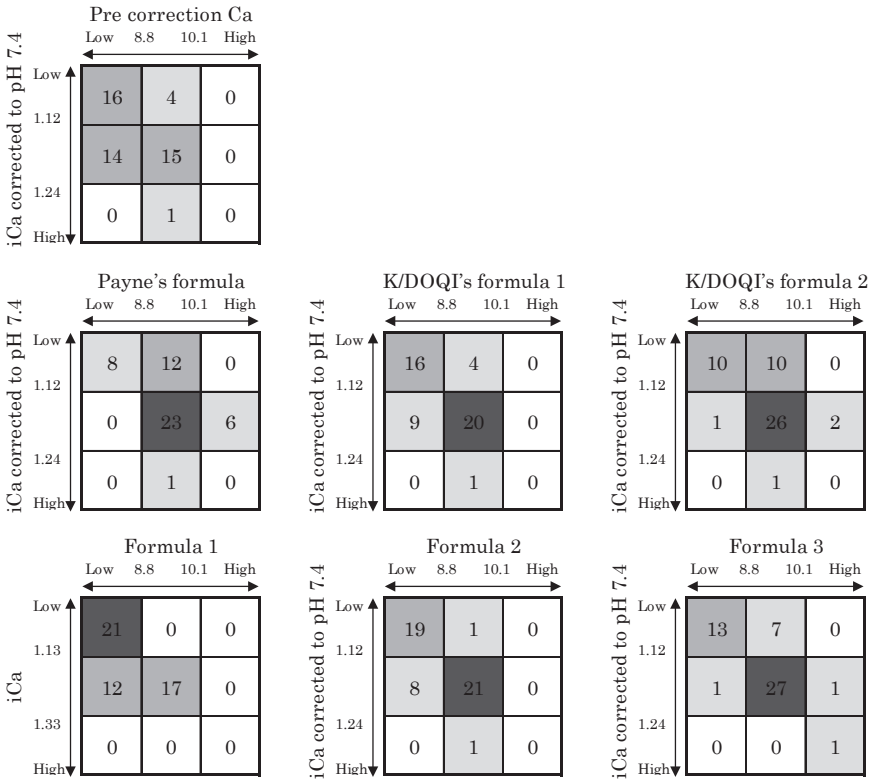

Figure 2 Classification of hemodialysis patients according to iCa or iCa corrected to pH 7.4. Based on iCa, 21 patients were classified as hypocalcemic (<1.13 mmol/L) and 29 patients were classified as normocalcemic. In contrast, based on iCa corrected to pH 7.4, 20 patients were classified as hypocalcemic (<1.12 mmol/L), 1 patient was classified as hypercalcemic and 29 patients were clas-sified as normocalcemic.

Table 2 Evaluation of the correction formulas for the determination of iCa and iCa corrected to pH 7.4

Ca formula		Sensitivity (%)	Specificity (%)	Agreement [1)]
Pre correction Ca	cCa (mg/dL) = tCa (mg/dL)	76.2	51.7	0.33
Payne's formula	cCa (mg/dL) = tCa (mg/dL) + 4 – Alb (g/dL)	38.1	79.3	0.43
K/DOQI's formula 1	cCa (mg/dL) = tCa (mg/dL) + 0.704(3.4 – Alb (g/dL))	76.2	69.0	0.48
K/DOQI's formula 2	cCa (mg/dL) = tCa (mg/dL) + 0.8(4 – Alb (g/dL))	47.6	89.7	0.49
Formula 1	ciCa (mmol/L) = 0.113tCa (mg/dL) – 0.338pH – 0.043Alb (g/dL) + 2.781	100.0	58.6	0.54
Formula 2	ciCa (mmol/L) = 0.116tCa (mg/dL) − 0.045Alb (g/dL) + 0.260	90.5	72.4	0.64
Formula 3	cCa (mg/dL) = tCa (mg/dL) + 0.4(4.6 – Alb (g/dL))	66.7	93.1	0.68

cCa: corrected Ca, tCa: total serum Ca, ciCa: corrected ionized calcium
Alb (g/L) = 10 × Alb (g/dL), Ca (mmol/L) = 0.2495 × Ca (mg/dL)
1) Weighted kappa coefficient, a value of 1.00 denotes perfect agreement

Sensitivity, specificity, and weighted kappa coefficient were evaluated using the data from 50 hemodialysis patients.

proteins [4)5)]. Although the BCP method has a high specificity to albumin, has been reported to underestimate the Alb concentration in patients with renal failure or dialysis [14)15)], and to react differently depending on the form of albumin, such as human mercaptalbumin (HMA) or human non-mercaptalbumin (HNA) [8)]. Therefore, the BCG and BCP methods may not be accurate enough to be used to precisely estimate iCa or cCa. In contrast, the mBCP resolves the difference in reactivity by adding sodium dodecyl sulfate and 5,5-dithiobis (2-nitrobenzoic acid), and achieves high specificity and stability to Alb [8)]. The Japanese Society for Dialysis Therapy guidelines recommends Payne's formula for patients with Alb concentration of less than 4.0 g/dL [16)], and the K/DOQI guideline also recommends that tCa concentration should be corrected when Alb concentration are low [3)]. In con-

trast, the reference interval of Alb determined by a large-scale study in Japan was 4.1 g/dL–5.1 g/dL, which is higher than these guidelines [13]. Collectively, this brings into question whether corrected Ca for a specific range of Alb concentration is appropriate for estimating or determining the iCa. In this study, we developed a new formula that can be corrected for all Alb concentration with iCa as the indicator using a mBCP method.

Three formulas were constructed using iCa or iCa corrected to pH 7.4 as an indicator and compared with the previous formulas using data from dialysis patients. Using Payne's formula, 20.7% of patients were judged to be hypercalcemic even though iCa corrected to pH 7.4 was at normal levels, and 60.0% of patients were failed to detect hypocalcemia, indicating that the correction using the mBCP method could not be used to evaluate Ca correctly with Payne's formula. In contrast, use of K/DOQI's formula 2 provided higher sensitivity, specificity, and agreement than Payne's formula, suggesting that it provides a result that is more reflective of the iCa condition than that obtained from Payne's formula. Formula 1, which directly estimates iCa, showed lower specificity and agreement than Formula 2 and Formula 3, albeit with higher sensitivity. Many formulas for estimating iCa concentration have been previously reported [17)-19)], but these formulas could not be used for comparison in this study because the reference interval differed depending on whether iCa was measured in serum [17)19)] or whole blood [18)]. Historically, it has been very difficult to calculate the iCa concentration accurately using only one formula because Ca homeostasis in the blood fluctuates depending on various factors such as pH, protein, parathyroid hormone, and calcitonin content [20)].

By comparison with Formula 1, Formula 2, which directly estimates iCa corrected to pH 7.4, and Formula 3, which was constructed from the relationship between the Ca reference interval and iCa corrected to pH 7.4, showed good specificity and agreement. In particular, Formula 3 provided superior sensitivity, specificity, and agreement with the results from Payne's formula and K/DOQI's formula 2, and showed the same level of agreement as Formula 2. This suggests that Formula 3 has the same performance as Formula 2, which can be used to directly estimate iCa. Furthermore, Formula 3 differs from formulas 1 and 2, which estimate iCa, in that it can be evaluated in terms of Ca concentration so it can be used in the same way as tCa is used in daily practice. Some formulas using the mBCP method have been previously reported [21)22)], none of them showed a better agreement than Formula 3 (Ohba's formula:0.49, Tanaka's formula:0.28).

Formula 3 uses iCa corrected to pH 7.4 instead of iCa as an index, and thus the iCa incorporated in the formula may be different from the actual in vivo iCa found in chronic kidney disease (CKD) patients with metabolic acidosis. The problem in CKD patients is that they suffer from hypocalcemia [3)]. The iCa concentration in patients with acidosis is higher than the iCa at pH 7.4 calculated by the pH adjustment formula. Therefore, unless hypocalcemia is determined using Formula 3, it is unlikely that the patient is actually hypocalcemic. Among the samples from the 50 hemodialysis patients analyzed in this study, there were seven patients whose iCa corrected to pH 7.4 was hypocalcemic but the Ca determined using Formula 3 was within the reference interval. Of these seven patients, three patients had acidosis and their iCa values were higher than the iCa corrected to pH 7.4, all within the reference interval of iCa. It is difficult to conclude which indicator is better to use to evaluate Ca in patients: iCa measured or iCa corrected to pH 7.4. However, it has been reported that iCa corrected to pH 7.4 is as useful as iCa measured in the evaluation of patients with chronic disorders of calcium metabolism [23)]. Therefo re, we consider that Formula 3 using iCa corrected to pH 7.4 as an indicator for correction is the most clinically practical, economic, and convenient method for Ca evaluation.

V. Conclusion

We developed Formulas 1, 2, and 3, which could be used across all Alb concentration, for more accurate estimation of iCa, iCa corrected to pH 7.4, and cCa, respectively. The formulas employed the mBCP method, which is the most accurate method for measuring the Alb concentration. On the basis that tCa is most commonly determined in the clinic, Formula 3 should be useful in daily practice even though its performance was essentially the same as that of Formula 2. We believe that the three new formulas will enable more accurate assessment of Ca conditions in the clinic.

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Author contributions

All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests

Authors state no conflict of interest.

Informed consent

Informed consent was obtained from all hemodialysis patients in this study. Owing to the retrospective nature of the study, informed consent was not required for the other patients.

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Original

Association between *delta opioid receptor* gene polymorphisms and alcohol dependence in a Japanese archipelago population

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ABSTRACT

Alcohol dependence (AD) is known to be a gene-related disease.

There are three types of opioid receptors, μ (mu), δ (delta), and κ (kappa). All three are thought to be associated with AD. The delta opioid receptors are associated with alcohol mediated processes in the brain.

In this study, we focused on the several known *OPRD1* (*opioid receptor delta 1*) gene polymorphisms that have not yet been the target of AD-related studies in a Japanese population, and that we can examine by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. we examined whether *OPRD1* gene polymorphisms (rs678849 and rs2234918) affected alcohol dependence development in 64 patients and 75 healthy people as controls. We also focused on an *ALDH2* gene polymorphism (rs671) as to restriction constitution. This analysis was also performed for the group with *ALDH2*1/*1*.

There were significant differences in carriers (major allele homozygous carriers versus minor allele carriers) of rs678849 between AD patients and controls (p=0.029), and also in the group with *ALDH2*1/*1* (p=0.0285). However, the significant differences were lost on the Bonferroni correction. There were no significant differences in rs2234918 between AD patients and controls. The haplotype analysis revealed there were no significant differences between AD and controls for the four haplotypes. On LD analysis, D' and r^2 were both found to be low (D'=0.31, r^2=0.022).

We concluded that the *OPRD1* gene polymorphisms rs678849 and rs2234918 might not be associated with alcohol dependence in a Japanese population. It is still necessary to analyze the *opioid receptor* gene in a larger sample size than that in this study.

〔Lab Med Int 2023; 2(2): 25-29〕

Key Words
delta opioid receptor, polymorphism, haplotype, linkage disequilibrium, alcohol dependence

I. Introduction

Alcohol dependence (AD) is known to be a gene-related disease.

There are three types of opioid receptors, μ (mu), δ (delta) and κ (kappa). The δ opioid receptors are associated with alcohol-mediated processes in the brain [1]. The previous study showed that the density of δ opioid receptors was significantly higher in the ventral tegmental area and nucleus accumbens of high alcohol preference mice [2]. These brain areas are related to the reward system. Furthermore, μ and δ opioid receptor antagonists reduce alcohol craving and consumption [3)-6)]. Thus, δ opioid receptors might be related to AD. δ opioid receptors are coded by the *OPRD1* (opioid receptor delta 1) gene, which is located at 1p36.1-p34.3 [3], spanning approximately 60 kb [7], and consisting of 3 exons [8].

In this study, we focused on the several known *OPRD1* gene polymorphisms that have not yet been the target of AD-related studies in a Japanese population, and that we can examine by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. We investigated whether the *OPRD1* gene polymorphisms rs678849 and rs2234918 are susceptibility factors for AD. rs678849 and rs2234918 are located in intron 1 and exon 3 on the *OPRD1* gene [3], as shown in **Figure 1**. In addition, the previous study revealed that rs678849 was significantly associated with regional frontal, temporal, and occipital brain volume [9]. rs678849 minor allele carriers had lower brain tissue volumes around the brain regions mentioned above in both the elderly group and the young healthy group [9]. rs2234918 (Gly307Gly) is a silent (synonymous) mutation would not change the composition of the proteins encoded by genes [3)10)]. It was reported that the silent SNP of the other gene is related to AD ($p = 0.003$) [11]. These SNPs are in dbSNP database (https://www.ncbi.nlm.nih.gov/snp/) [19)20)].

The *ALDH2* gene polymorphism (rs671:1510G/A) 1510G allele was significantly associated with AD in a Japanese population [12]. 1510A allele carriers (1510G/A and 1510A/A) are flushers who experience reactions such as nausea, palpitations, and headaches caused by drinking of a small amount of alcohol. Accordingly, we also performed analysis limited to the 1510G/G (*ALDH2**1/*1) carriers among the subjects to focus on the restriction constitution.

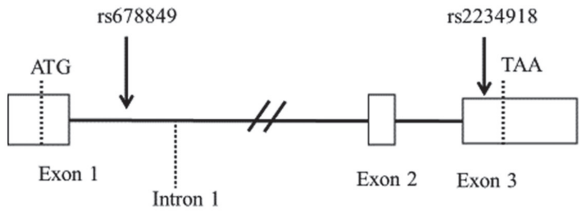

Figure 1 Polymorphism positions in the *OPRD1* gene.

II. Material and Methods

1. Subjects

This study was approved by the Ethics Committee of Azabu University, Japan (2359). Written informed consent was obtained from the 64 AD patients (57 males; 7 females) diagnosed according to DSM-IV diagnostic criteria and 75 healthy people (23 males; 52 females) as controls.

2. DNA Analysis

The two *OPRD1* gene polymorphisms were examined by means of PCR-RFLP according to the methods of Zhang et al. (2008) [3] and Gelernter and Kranzler. (2000) [13] The *ALDH2* gene polymorphism was examined by the method of Wu et al. (2005) [14]. The PCR conditions for the T100 Thermal Cycler (Bio-Rad Laboratories, Inc. Hercules, CA) were as follows: rs678849 (Initial denaturation for 5 min at 95℃ , followed by 35 cycles of denaturation for 30 s at 95℃ , annealing for 30 s at 53℃ , and extension for 30 s at 72 ℃ , followed by final extension for 5 min at 72℃); rs2234918 (As for rs678849 except for the annealing temperature, 65℃); and rs671 (Initial denaturation for 10 min at 95℃ , followed by 35 cycles of denaturation for 30 s at 95℃ , annealing for 30 s at 60℃ , and extension for 30 s at 72 ℃ , followed by final extension for 7 min at 72℃). The PCR products were digested with *Rsa*I (Nippongene, Tokyo, Japan), *Alu*I (Takara, Shiga, Japan) or *Mbo*II (New England Biolabs, Tokyo, Japan). The digested products were subjected to electrophoresis on agarose gels using the ethidium bromide staining method, as shown in **Figure 2**.

3. Statistical Analyses

The Hardy-Weinberg disequilibrium was assessed using a chi-square test. We compared the *OPRD1* genotypes, alleles, and carriers (minor allele carriers versus major allele homozygotes carriers) between AD patients and controls by performing statistical analysis using a chi-square test with Yate's correction. These statistical analyses were performed using ystat2018 [15]. Haplotype frequencies and linkage disequilibrium (LD) coefficients were calculated with gPLINK v. 2.050 (http://zzz.bwh.harvard.edu/plink/index.shtml) as described by Purcell S et al. (2007) [16], and Haploview v.4.2 (http://www.broad.mit.edu/mpg/haploview/index.php) [17]. A p-value less than 0.05 was considered statistically significant in this study. The Bonferroni correction was applied to correct for mul-

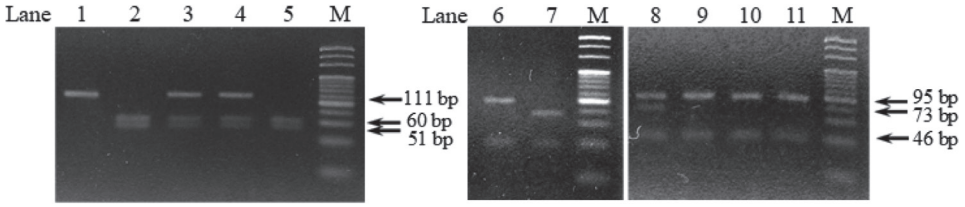

Figure 2 Ethidium bromide stained 4% agarose gel illustrating the *Rsa* I or the *Alu* I restriction fragments of *OPRD1* gene polymprphism rs678849 or rs2234918. rs678849: Lane M, 20 bp DNA Ladder; Lane1, C/C eggnotype; Lane 2 and 5, T/T genotype; Lane 3 and 4, T/C genotype. rs2234918: Lane M, 20 bp DNA ladder; Lane 6,9,10 and 11, T/T genotype; Lane 7, C/C genotype, Lane 8, T/C genotype. (22 bp was not visible.)

tiple comparisons, the p-value was adjusted.

III. Results

Table 1 shows that the two *OPRD1* gene genotype, allele, and carrier (minor allele carriers versus major allele homozygotes carriers) frequencies in AD patients and controls. The genotypes frequencies were as follows: rs678849 (Alcohol + Control; T/T: 16 + 32, T/C: 38 + 31, C/C: 10 + 12); and rs2234918 (T/T: 50 + 55, T/C: 13 + 18, C/C: 1 + 2). The genotype distribution was in Hardy-Weinberg equilibrium (data not shown). Although no significant differences in the genotype and allele frequencies of rs678849 were found (genotypes: $\chi^2(2) = 5.389, p = 0.0676$; alleles: $\chi^2(1) = 2.140, p = 0.144$), there was significant difference in the carrier (major allele homozygous carriers versus minor allele carriers) frequency between AD patients and controls (carriers (T/C + C/C vs T/T): $\chi^2(1) = 4.767, p = 0.029$). However, the significant difference was lost on the Bonferroni correction (The Bonferroni p-value was adjusted 0.05/2 = 0.025). There were no significant differences for rs2234918 (genotypes: $\chi^2(2) = 0.148, p = 0.929$; alleles: $\chi^2(1) = 0.520, p = 0.471$; carriers (T/C + C/C vs T/T): $\chi^2(1) = 0.429, p = 0.512$). In addition, we also compared *ALDH2**1/*1 carriers between AD patients and controls. **Table 2** shows there were also significant differences in rs678849 carrier frequency in *ALDH2**1/*1 carriers between AD patients and controls (carriers (T/C + C/C vs T/T) $\chi^2(1) = 6.644, p = 0.0285$). However, the significant difference was lost on the Bonferroni cor-

Table 1 Genotypes and frequencies of the polymorphisms of the *OPRD1* gene in AD subjects and controls.

SNP	Subject	n	Genotype (%)			Allele (%)		Carrier (%)	
			T/T	T/C	C/C	T	C	T/T	T/C + C/C
rs678849	AD	64	16 (25.0)	38 (59.4)	10 (15.6)	70 (54.7)	58 (45.3)	16 (25.0)	48 (75.0)
	Control	75	32 (42.7)	31 (41.3)	12 (16.0)	95 (63.3)	55 (36.7)	32 (42.7)	43 (57.3)
			$\chi^2(2)=5.389$ $p=0.0676$			$\chi^2(1)=2.140$ $p=0.144$		$\chi^2(1)=4.767$ $p=0.029$*	
rs2234918			T/T	T/C	C/C	T	C	T/T	T/C + C/C
	AD	64	50 (78.1)	13 (20.3)	1 (1.6)	113 (88.2)	15 (11.7)	50 (78.1)	14 (21.9)
	Control	75	55 (73.3)	18 (24.0)	2 (2.7)	128 (85.3)	22 (14.7)	55 (73.3)	20 (26.7)
			$\chi^2(2)=0.148$ $p=0.929$			$\chi^2(1) = 0.520$ $p=0.471$		$\chi^2(1)=0.429$ $p=0.512$	

Carrier (%): major allele homozygous carriers vs minor allele carriers (%).
*p<0.05 (exact significance, chi-square test).

Table 2 Genotypes and frequencies of the polymorphisms of the *OPRD1* gene in AD subjects and controls in *ALDH2*1/*1* carriers.

SNP	Subject	n	Genotype (%)			Allele (%)		Carrier (%)	
			T/T	T/C	C/C	T	C	T/T	T/C + C/C
rs678849	AD	64	16 (25.8)	36 (58.1)	10 (16.1)	68 (54.8)	56 (45.2)	16 (25.8)	46 (74.2)
	Control	48	22 (45.8)	19 (39.6)	7 (14.6)	63 (65.6)	33 (34.4)	22 (45.8)	26 (54.2)
			$\chi^2(2)=4.087$ $p=0.130$			$\chi^2(1)=2.61$ $p=0.106$		$\chi^2(1)=6.644$ $p=0.0285$*	
rs2234918			T/T	T/C	C/C	T	C	T/T	T/C + C/C
	AD	62	49 (79.0)	12 (19.4)	1 (1.6)	110 (88.7)	14 (11.3)	49 (79.0)	13 (21.0)
	Control	48	35 (72.9)	13 (27.1)	0 (0)	83 (6.5)	13 (13.5)	35 (72.9)	13 (27.1)
			$\chi^2(2)=0.493$ $p=0.782$			$\chi^2(1) = 0.255$ $p=0.614$		$\chi^2(1)=0.561$ $p=0.454$	

Carrier (%): major allele homozygous carriers vs minor allele carriers (%).
*p<0.05 (exact significance, chi-square test).

Table 3 Haplotype frequencies in the *OPRD1* gene polymorphisms in AD and controls.

Haplotype	rs678849	rs2234918	Frequency		χ^2	Haplotype
			AD	Control		*p* value
1	T	C	0.0365	0.0699	1.495	0.2215
2	T	T	0.5104	0.5634	0.7825	0.3764
3	C	C	0.0807	0.0768	0.014660	0.9036
4	C	T	0.37240	0.28990	2.135	0.144

Haplotype distributions were not significantly different between AD and controls (global p = 0.3586).

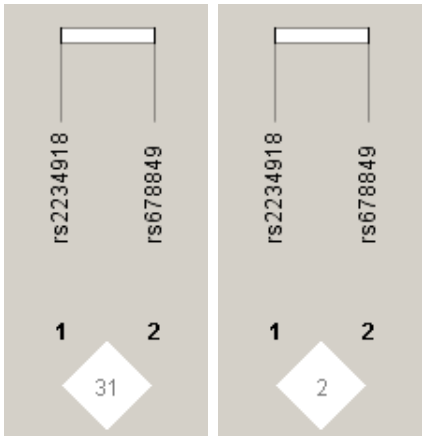

Figure 3 Linkage disequilibrium (LD) map of the *OPRD1* locus examined in this study.
(a) *D'* value = 0.31, (b) r^2 value = 0.022.

rection. There were no significant differences in the genotype and allele frequencies of rs678849 and rs2234918 (rs678849-genotypes: $\chi^2(2) = 4.087, p = 0.130$; alleles: $\chi^2(1) = 2.61, p = 0.106$; rs2234918-genotypes: $\chi^2(2) = 0.493, p = 0.782$; alleles: $\chi^2(1) = 0.255, p = 0.614$; carriers (T/C + C/C vs T/T): $\chi^2(1) = 0.561, p = 0.454$). Haplotype analysis revealed there were no significant differences between AD patients and controls for the four haplotypes, as shown in **Table 3** (global p-value = 0.3586). On LD analysis, *D'* and r^2 were both found to be low, as shown in **Figure 3** ($D' = 0.31, r^2 = 0.022$).

IV. Discussion

The results suggest that the *OPRD1* gene polymorphism rs678849 and rs2234918 might not be associated with AD in a Japanese population in this study.

However, it might be some study limitations in this study.

Firstly, we should to consider statistics. The significant differences of rs678849 in carriers were lost on the Bonferroni correction. The Bonferroni correction can reduce the chance of a type I error but at the expense of a type II error[18]. We might want to consider about a type II error that produces a false negative.

Secondly, we should focus on subjects. Using a sample size larger than this subject might reveal the significant differences. As a side note, there were not found significant differences of allele frequency in both Asian population and this control subject. The T and C carriers of rs678849 were found 62.7% and 37.3% in 750 Asian population [19]. The T and C carriers of rs2234918 were found 82.0% and 18.0% in 172 Asian population [20]. Besides, the T and C carriers of rs678849 were found 63.3%and 36.7%, the T and C carriers of rs2234918 were found 85.3% and 14.7% in 75 Japanese control subjects.

In addition, the difference between AD patients and controls was expected to become further apparent by limiting examination to *ALDH2**1/*1 (restricting constitution). The relationships were confirmed both in all subjects and in a group with *ALDH2**1/*1, there were not found significant differences of the results.

In the haplotype analysis results, there were no associations of the four haplotypes with each other. In the previous study on a European American population, there was significant association of the haplotype including rs2234918 with AD [3]. Therefore, other SNPs also should be considered in AD-related studies like the previous study [3]. Regarding the LD analysis, it is unlikely that there is an LD between rs678849 and rs2234918. However, this study does not cover all LD blocks [7].

V. Conclusion

There have been few reports of association studies between *OPRD1* gene polymorphisms and AD in the world.

This study was an exploratory study in a Japanese archipelago population. Therefore, it is need to consider study limitations as mentioned above, further research with a large sample size is needed to draw conclusions. Through this, the relationship between opioid receptors genes polymorphisms and AD might be further revealed.

Disclosure

No potential conflicts of interest were disclosed.

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Original

Findings on conventional sonography to predict the presence of liver injury in elderly women with non-alcoholic fatty liver disease

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ABSTRACT

Introduction: Patients with non-alcoholic fatty liver disease (NAFLD) often demonstrate liver injury, as reflected by an elevated serum level of alanine aminotransferase (ALT). This study examined whether the presence of liver injury could be predicted based on findings from conventional sonography.
Methods: Subjects were 81 adult female NAFLD patients (mean age, 62 ± 14 years). Defining liver injury as ALT levels >30 IU/L, sonographic findings were compared between patients with and without liver injury. In turn, ALT levels and liver size (defined as the sum of the length of the right lobe [R1+R2] and left lobe [L1+L2] were compared among the three classical grades of fatty liver by sonography.
Results: Grade 3 fatty liver, deep attenuation, and hepatomegaly demonstrated relatively high odds ratios for the presence of liver injury. Median L1+L2 and R1+R2+L1+L2 were significantly longer in patients with liver injury (164 mm [interquartile range, 149–178 mm] and 289 mm [267–314 mm]) than in those without (147 mm [130–156 mm] and 260 mm [247–281 mm], respectively; $p<0.001$ each). ALT levels, L1+L2, and R1+R2+L1+L2 increased with increasing fatty liver grade.
Conclusion: The present study suggests that grade 3 fatty liver, deep attenuation and/or hepatomegaly allow easy, non-invasive prediction of the presence of liver injury in elderly women with NAFLD using conventional sonography.

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Key Words
aminotransferase, hepatomegaly, liver injury, non-alcoholic fatty liver disease (NAFLD), sonography

I. Introduction

Non-alcoholic fatty liver disease (NAFLD) is considered a hepatic manifestation of metabolic syndrome[1)-3)]. Patients with NAFLD often demonstrate liver injury, clinically recognizable as an elevated serum level of alanine aminotransferase (ALT)[1)4)]. ALT is an enzyme released into the blood when hepatocytes are injured.

Persistent liver injury can lead to hepatic fibrosis as an attempted restorative process in the tissue, eventually leading to liver cirrhosis[1] and occasionally the development of liver cancer[5]. The presence of liver injury also correlates with the risk of atherosclerosis in patients with NAFLD[6)7)]. Since the initial diagnosis of fatty liver is often made from abdominal sonography because of the availability, low cost, and non-invasiveness of this modality[8)9)], the ability to use sonographic data to predict the presence of liver injury would be particularly convenient. However, the relationship between liver injury and sonographic findings of fatty liver has yet to be clarified. Meanwhile, a new technique of sonography, the controlled attenuation parameter (CAP) using vibration-controlled transient elastography (VCTE), has recently been utilized to assess the grade of hepatic steatosis[2)3)10)11)]. Another recent advance in evaluating hepatic steatosis is the attenuation coefficient (AC) using attenuation imaging (ATI), developed as two-dimensional B-mode sonography[12)13)]. Not every medical setting, however, has access to such advanced modalities. The present study therefore attempted to elucidate whether the presence of liver injury in patients with NAFLD could be predicted using commonly available conventional sonography and what findings may hold promise for such prediction.

II. Methods

1. Subjects

Subjects comprised 81 female outpatients at our hospital (mean age, 62 ± 14 years) with lifestyle-related diseases such as dyslipidemia, diabetes, and hypertension who had been diagnosed with fatty liver on abdominal sonography. Dyslipidemia was defined as having laboratory abnormalities of low-density lipoprotein cholesterol (>140 mg/dL), high-density lipoprotein cholesterol (<36 mg/dL) or triglycerides (>130 mg/dL). Diabetes was defined as having laboratory abnormalities of hemoglobin A1c (>5.8%). Hypertension was defined as abnormalities of systolic blood pressure (≥ 140 mmHg) or diastolic blood pressure (≥ 90 mmHg) at the time of consultation as an outpatient. Hyperuricemia was defined as having laboratory abnormalities of uric acid (>7.0 mg/dL). Patients who had already been treated with medications for these diseases were also included as patients having these lifestyle-related diseases, even if laboratory examination values were within normal ranges. Patients with habitual ethanol intake (>10 g/day), a history of seropositivity for hepatitis B or C virus, autoimmune disease, malignant tumors, or the use of medications that may modulate the results of blood tests for liver function were excluded from this study. Patients suspected as having liver fibrosis or chronic renal dysfunction were also excluded on the basis of blood test results and sonographic findings[14)]. The present study therefore dealt with subjects considered to be showing early-stage NAFLD, not advanced-stage NAFLD such as liver cirrhosis.

Sonography

All sonographic examinations were performed using an Aplio ultrasound scanner (Canon Medical Systems Corporation, Tochigi, Japan) equipped with 3.5-MHz convex-array transducers. Fatty liver was diagnosed with high accuracy using conventional sonography, taking advantage of the 85% sensitivity and 94% specificity for detecting moderate to severe hepatic steatosis[8)]. First, sonographic findings in patients were evaluated in terms of 'fatty liver grade', classified by a classical method into one of three grades[15)-17)]. These three grades have been described on the basis of imaging differences, as follows: grade 1 (mild), slightly diffuse increase in echogenicity of liver parenchyma with normal visualization of the diaphragm and intrahepatic vessel borders; grade 2 (moderate), a moderate, diffuse increase in the echogenicity of liver parenchyma with slightly impaired visualization of intrahepatic vessels and diaphragm; and grade 3 (severe), a marked increase in the echogenicity of liver parenchyma with poor or absent visualization of intrahepatic vessels, diaphragm and posterior segment of the right lobe of the liver. Second, the presence or absence of blurred vessels, deep attenuation, focal spared areas and hepatomegaly were evaluated[1)17)]. At this time, hepatomegaly was defined as poor visualization of posterior segment of the right lobe of the liver in subcostal scans. Hepatomegaly was therefore the same as one of the diagnostic criteria for grade 3 fatty liver. Third, liver size was quantitatively measured by applying the method used for measuring liver size in children[18)19)], since 'hepatomegaly' could be a qualitative evaluation by the sonographer. The measurement method was as follows: lengths from the midpoint of the horizontal portion of the portal vein to the anterior surface of the liver (R1) and to the deepest phrenic surface of the liver (R2) were measured from the right subcostal scan in sonography (**Figure 1**), and R1+R2 was used as a marker representing hepatic right lobe size. Lengths between the top and bottom (L1), and between the dorsal and ventral margins (L2) were then measured from the mid-longitudinal scan in sonography, and L1+L2 was used as a marker representing hepatic left lobe size. The sum of R1+R2 and L1+L2 was then taken as the measured liver size. A swollen right lobe such as observed in grade 3 fatty

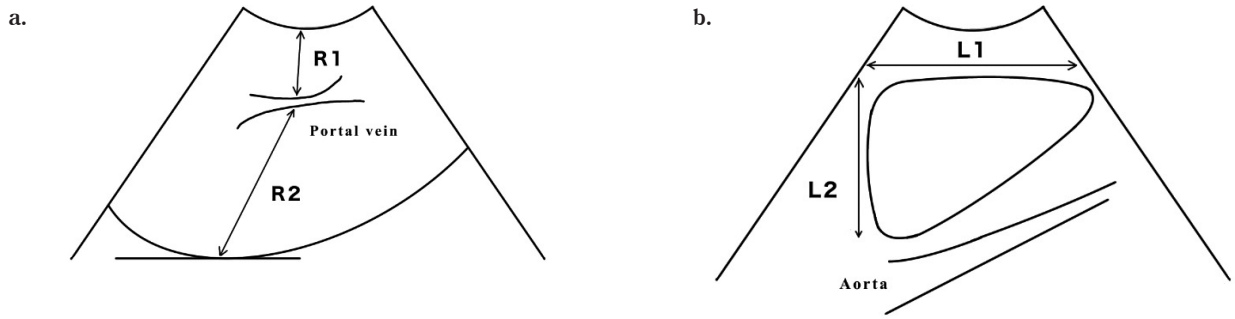

Figure 1 Measurement of liver size

a) Lengths from the midpoint of the horizontal portion of the portal vein to the anterior surface of the liver (R1) and to the deepest phrenic surface of the liver (R2) on the right subcostal scan in sonography, for calculation of R1+R2 as a marker of hepatic right lobe size. **b**) Lengths between the top and bottom (L1) and between the dorsal and ventral margins (L2) on the mid-longitudinal scan in sonography, for calculation of L1+L2 as a marker of hepatic left lobe size.

liver was not entirely visualized in the field of subcostal scanning for adults, since this measurement method has been established for measurement in children. In such cases, length from the portal horizontal portion to the bottom of scanning field was adopted as R2. Last, spleen size was assessed by calculating spleen index, as well as by measuring maximal length of the spleen[20]. All evaluations concerning fatty liver grade and the presence or absence of blurred vessels, deep attenuation, focal spared areas, and hepatomegaly were performed independently by one medical doctor specializing in sonography and two skilled sonographers for each image. Evaluations were performed under blinded conditions, with no investigator aware of patient clinical histories. Mean results were adopted to minimize any observer bias.

2. Blood examination

Blood tests performed on the days of sonographic examinations were evaluated, including liver function tests such as aspartate aminotransferase (AST), γ-glutamyl transpeptidase (GGT), alkaline phosphatase, and ALT. Blood tests also included those related to the severity of lifestyle-related diseases, such as triglycerides, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, glucose, hemoglobin A1c, and uric acid levels. Platelet count was also assessed to exclude patients who had already developed hepatic fibrosis[1)14)21)]. AST-to-ALT ratio (AST/ALT ratio) was also calculated, since an increased value for this ratio to >1 suggests hepatic fibrosis[22)23)]. These values were used because sonography is not good at detecting hepatic steatosis when advanced fibrosis is present in the liver[11)].

3. Analyses

Patients were divided into two groups according to the presence (ALT >30 IU/L, upper limit of the reference range) or absence of liver injury. Clinical features, blood examination results and sonographic findings from patients were compared between these two groups. Since some data showed non-normal distributions using the Shapiro-Wilk test, descriptive results are presented as median values with interquartile ranges (IQRs) in the text and as box-and-whisker plots of medians with quartiles and minimum and maximum values in the figures. To assess the significance of differences in sonographic findings to predict the presence of liver injury, the Mann-Whitney test was applied to compare the groups with and without liver injury for continuous data, and Pearson' s chi-square test or Fisher' s exact probability test for contingency table data. When a significant finding was detected ($p<0.05$) for contingency table data, adjusted residuals were then analyzed to confirm the significance of the finding for the presence or absence of liver injury. At this time, adjusted residuals >1.96 and >2.58 were considered to correspond to significant values of $p<0.05$ and $p<0.01$, respectively. To identify findings predicting the presence of liver injury, the odds ratio of each sonographic finding for the presence of liver injury was calculated. For measured liver size, the cut-off values of R1+R2, L1+L2, and R1+R2+L1+L2 for detecting serum ALT abnormality as determined using receiver operating characteristic (ROC) analyses were employed as criteria. Odds ratios for the presence of liver injury were also assessed according to the presence of each lifestyle-related disease. Next, ALT levels and liver size measured as R1+R2, L1+L2, or R1+R2+L1+L2 on sonography which had exhibited significant differences between patients with and without liver injury, were compared among the three grades of fatty liver. The Kruskal-Wallis test and subsequent Bonferroni correction for multiple comparisons were used for comparisons among the three fatty liver grades. Values of $p<0.05$ after Bonferroni correction were considered statistically significant.

Informed consent for sonography and blood examination was obtained from each patient. All study protocols were approved by the ethics committee of our institute (approval number: 348). All procedures were performed in accordance with the ethical standards formulated in the Declaration of Helsinki and its revisions.

III. Results

The clinical features of patients are presented in **Table 1**. Age was significantly lower in patients with liver injury (61 years, IQR 48–69 years) than in those without (63 years, IQR 59–74 years; p=0.039). Presence of lifestyle-related diseases did not differ significantly between

Table 1 Comparison between patients with and without liver injury.

			With liver injury n=34	Without liver injury n=47	
Clinical features					p
Age	years		61 [48–69]	63 [59–74]	0.039 †
Dyslipidemia	Present/absent		30/4	39/8	0.511
Diabetes	Present/absent		25/9	33/14	0.744
Hypertension	Present/absent		17/17	21/26	0.636
Hyperuricemia	Present/absent		10/24	10/37	0.402
Blood examinations	Units	Reference range			p
AST	IU/L	8–33	39 [30–50]	19 [16–23]	0.000 †
ALT	IU/L	3–30	49 [37–67]	20 [15–23]	0.000 †
AST/ALT ratio			0.70 [0.64–0.89]	1.05 [0.91–1.18]	0.000 †
GGT	IU/L	4–50	55 [31–114]	24 [19–36]	0.000 †
Alkaline phosphatase	IU/L	108–313	234 [224–317]	230 [197–287]	0.371
Triglycerides	mg/dL	60–130	137 [114–208]	134 [98–173]	0.308
HDL-cholesterol	mg/dL	36–70	58 [49–68]	56 [49–65]	0.778
LDL-cholesterol	mg/dL	–140	114 [98–146]	118 [96–131]	0.954
Glucose	mg/dL	60–110	122 [100–153]	114 [99–127]	0.297
Hemoglobin A1c	%	4.6–6.2	6.3 [5.7–7.9]	6.2 [5.5–8.0]	0.560
Uric acid	mg/dL	2.0–7.0	5.3 [4.6–6.1]	4.7 [4.3–5.5]	0.068
Platelet count	$\times 10^4/\mu L$	15.0–38.0	22.4 [19.5–24.9]	21.4 [18.1–25.0]	0.562
Sonographic findings					
Grade	3/2/1 (adjusted residuals)		6*/19/9 (2.5)	1/17/29* (3.1)	0.002 †
Blurred vessels	Present/absent (adjusted residuals)		28*/6 (2.4)	27/20* (2.4)	0.018 †
Deep attenuation	Present/absent (adjusted residuals)		7*/27 (2.7)	1/46* (2.7)	0.008 †
Focal spared areas	Present/absent		12/22	17/30	0.935
Hepatomegaly	Present/absent (adjusted residuals)		20*/14 (5.2)	3/44* (5.2)	0.000 †
	Units				
R1+R2	mm		125 [118–138]	122 [114–128]	0.066
L1+L2	mm		164 [149–178]	147 [130–156]	0.000 †
R1+R2+L1+L2	mm		289 [267–314]	260 [247–281]	0.000 †
Splenic long axis	mm		95 [87–102]	89 [77–99]	0.144
Spleen index	mm^2		3104 [2581–3382]	2632 [2100–3278]	0.088

Results are presented as median [interquartile range] for continuous data, and as the number of patients with or without (present/absent) each sonographic finding.
Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyl transpeptidase; HDL, high-density lipoprotein; LDL, low-density lipoprotein. † p<0.05, Mann-Whitney U-test, Pearson's chi-square test, or Fisher's exact probability test. *p<0.05, adjusted residuals. Significance of adjusted residuals is shown for the presence of each sonographic finding, not the absence, to characterize presence or absence of liver injury. Adjusted residuals >1.96 and >2.58 were taken to correspond to statistically significant values of p<0.05 and p<0.01, respectively.

groups. Concerning laboratory data other than ALT, both AST and GGT levels were significantly higher in patients with liver injury than in those without. The ratio of AST to ALT in patients with liver injury was less than 1 (0.70, IQR 0.64–0.89), whereas that in patients without liver injury was more than 1 (1.05, IQR 0.91–1.18). No differences in blood examination findings related to lifestyle-related diseases were seen between patients with and without liver injury. Platelet counts likewise did not differ between groups and were within normal range (liver injury: 22.4 $\times 10^4/\mu L$, IQR 19.5–24.9 $\times 10^4/\mu L$; without liver injury: 21.4 $\times 10^4/\mu L$, IQR 18.1–25.0 $\times 10^4/\mu L$; p=0.562).

As for sonographic findings, grade 2 or 3, blurred vessels, deep attenuation, and hepatomegaly were more frequent in patients with liver injury than in those without liver injury (**Table 1**). Grade 3 fatty liver was a significant finding for confirming the presence of liver injury (adjusted residuals 2.5, corresponding to $p<0.05$), while grade 1 fatty liver was for confirming the absence of liver injury (adjusted residuals 3.1, corresponding to $p<0.01$). Blurred vessels (adjusted residuals 2.4, corresponding to $p<0.05$), deep attenuation (adjusted residuals 2.7, corresponding to $p<0.01$) and hepatomegaly (adjusted residuals 5.2, corresponding to $p<0.01$) were also significantly associated with the presence of liver injury. L1+L2 was significantly longer in patients with liver injury (164 mm, IQR 149–178 mm) than in patients without (147 mm, IQR 130–156 mm; $p<0.001$), although R1+R2 did not differ significantly between groups. R1+R2+L1+L2 was also significantly longer in patients with liver injury (289 mm, IQR 267–314 mm) than in patients without (260 mm, IQR 247–281 mm; $p<0.001$). In contrast to liver size, spleen size did not differ between groups.

The cut-off values of L1+L2 and R1+R2+L1+L2 for detecting serum ALT abnormality from ROC analyses were 162 mm (area under the curve [AUC] 0.759) and 260 mm (AUC 0.746), respectively. According to odds ratios, patients showing grade 3 fatty liver, deep attenuation, hepatomegaly, L1+L2 >162 mm, and R1+R2+L1+L2 >260 mm were suggested to show a relatively high risk of liver injury (**Table 2**). In contrast, the presence of lifestyle-related diseases was not associated with the presence of liver injury in patients with NAFLD.

Serum ALT levels increased with increasing fatty liver grade (grade 1: 22 IU/L, IQR 18–30 IU/L; grade 2: 32 IU/L, IQR 20–62 IU/L; grade 3: 56 IU/L, IQR 43–87 IU/L) (**Figure 2**). Liver size as measured by L1+L2 (grade 1: 144 mm, IQR 128–154 mm; grade 2: 155 mm, IQR 147–168 mm; grade 3: 190 mm, IQR 173–200 mm) and R1+R2+L1+L2 (grade 1: 256 mm, IQR 244–278 mm; grade 2: 279 mm, IQR 265–294 mm; grade 3: 320 mm, IQR 306–334 mm) also increased with increasing fatty liver grade (**Figure 3**).

IV. Discussion

This is the first study to examine whether the presence of liver injury can be predicted in elderly women with

Table 2 Odds ratio of each sonographic finding for the presence of liver injury

	Odds ratio	95% confidence interval	
Sonographic findings			
Grade 2	3.60	1.33–9.73	*
Grade 2 or 3	4.48	1.71–11.7	*
Grade 3	19.33	2.05–182.55	*
Blurred vessels	3.46	1.20–9.92	*
Deep attenuation	11.93	1.39–102.23	*
Focal spared areas	0.96	0.38–2.42	
Hepatomegaly	20.95	5.41–81.17	*
R1+R2 >133 mm	3.73	1.23–11.29	*
L1+L2 >162 mm	6.18	2.23–17.10	*
R1+R2+L1+L2 >260 mm	7.83	2.38–25.72	*
Complications			
Dyslipidemia	1.54	0.42–5.60	
Diabetes	1.18	0.44–3.16	
Hypertension	1.24	0.51–3.00	
Hyperuricemia	1.54	0.56–4.26	

* significant, $p<0.05$.

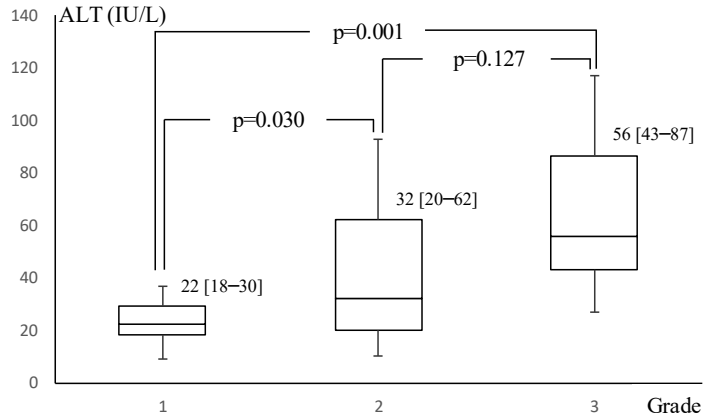

Figure 2 Serum alanine aminotransferase (ALT) levels by fatty liver grade

The upper limit of the normal range for ALT is 30 IU/L. Box-and-whisker plots show an increase in serum alanine aminotransferase (ALT) levels with each fatty liver grade (grade 1: n=38; grade 2: n=36; grade 3: n=7). The bottom and top of each box represent the 25th and 75th percentiles, respectively. The line through the box denotes the median, and the whiskers denote the minimum and maximum values. Values are presented as medians with quartiles. Bonferroni correction for multiple comparisons was used for comparisons among the three fatty liver grades. These adjusted values of $p<0.05$ were considered statistically significant.

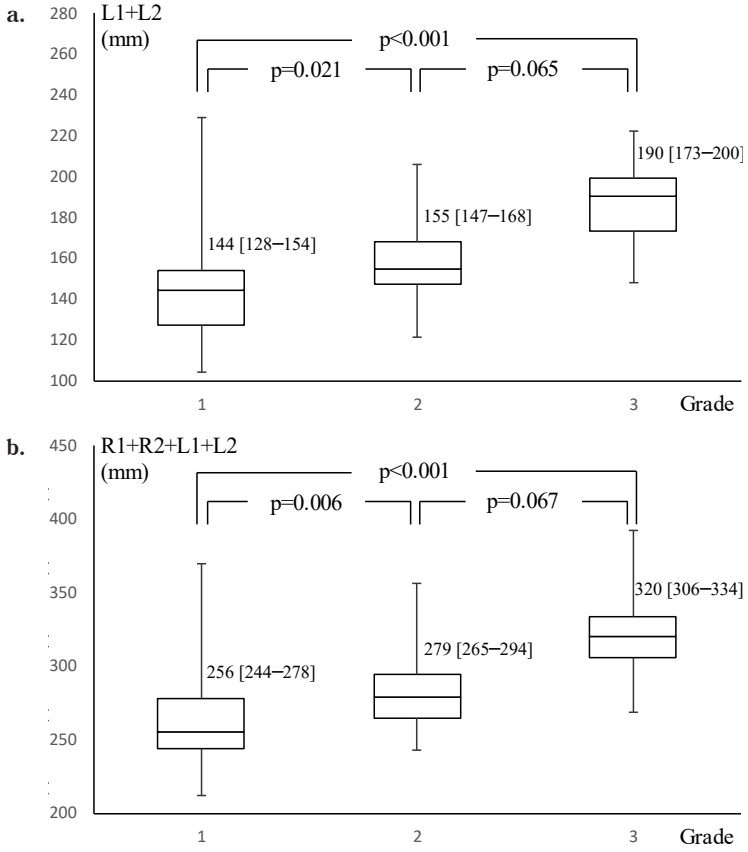

Figure 3 Length L1+L2 and R1+R2+L1+L2 by fatty liver grade

Box-and-whisker plots show increases in lengths L1+L2 (**a**) and R1+R2+L1+L2 (**b**) with each fatty liver grade (grade 1: n=38; grade 2: n=36; grade 3: n=7). The bottom and top of each box represent the 25th and 75th percentiles, respectively. The line through the box denotes the median, and the whiskers denote the minimum and maximum values. Values are presented as medians with quartiles. Bonferroni correction for multiple comparisons was used for comparisons among the three fatty liver grades. These adjusted values of $p<0.05$ were considered statistically significant.

NAFLD on the basis of findings from conventional sonography, and what findings could prove promising on examination. The present findings suggest fatty liver grade 3, deep attenuation, and hepatomegaly also represented by L1+L2 or R1+R2+L1+L2 as reliable candidate predictors of the presence of liver injury (**Tables 1, 2; Figures**

2, 3). The results also suggest that fatty liver grading by sonography is useful for predicting the severity of liver injury and hepatomegaly.

Grade 3 fatty liver is considered reflective of severe fat deposition in the liver[15)17)], which in turn causes hepatomegaly[24)] and deep attenuation on sonographic images. These three findings were thus essentially linked. Concerning liver size, grade 3 was actually more closely associated with larger liver size than the other grades of fatty liver on the measured scale (**Figure 3**). Among the criteria for diagnosis of grade 3[15)17)], increased echogenicity of liver parenchyma and impaired visualization of intrahepatic vessels and the diaphragm might not be readily distinguishable from the criteria for grade 2, depending on the subjective opinion of the observer. Conversely, the other criterion for diagnosis of grade 3, impaired visualization of the posterior right lobe, could be judged objectively since the posterior right lobe could not be visualized within the margin of the sonographic image due to hepatomegaly. Deep attenuation, the other candidate for predicting the presence of liver injury, also correlated with the finding of grade 3, since this impairs visualization of the posterior right lobe on sonography. Grade 3 demonstrating poor or absent visualization of the posterior segment of the right lobe due to hepatomegaly and/or deep attenuation could thus represent a promising finding for predicting the presence of liver injury on sonography. This conclusion may be consistent with results from magnetic resonance imaging (MRI) on pediatric patients with NAFLD, demonstrating correlations between serum ALT levels and hepatic fat content[25)-27)] or between serum ALT levels and liver volume[28)]. However, sonography offers advantages over MRI in terms of convenience and cost-benefit ratio.

Non-invasive imaging methods have been developed to evaluate the degree of hepatic steatosis, since liver biopsy, as a gold standard for diagnosing NAFLD, is an invasive procedure that is unlikely to be applied widely in clinical settings[9)11)]. Concerning sonography, a scoring system using sonographic findings has been reported[29)]. However, that system was established only for evaluating the degree of hepatic steatosis, not for predicting the presence of liver injury. Scoring using this method in daily clinical settings might also be somewhat complicated. In the present study, grade 3 fatty liver, deep attenuation and hepatomegaly demonstrated a high (>10 ×) odds ratio for the presence of liver injury, suggesting that these indices can be individually applied to select patients warranting precise investigations of liver injury.

Meanwhile, CAP from simultaneous VCTE and AC from ATI are recent advances in sonographic techniques, based on the process of measuring the degree of sonographic attenuation due to hepatic steatosis[2)3)10)-13)]. CAP and AC thus offer objective and precise imaging methods to noninvasively evaluate the degree of fatty liver. The FibroScan-AST score (FAST) can predict not only the degree of hepatic steatosis, but also the degree of hepatic fibrosis as a criterion for total staging of NAFLD patients[30)31)]. The presence or absence of liver injury in patients with NAFLD could therefore also be recognized by measuring CAP with FAST scoring, or AC from ATI. However, not every medical setting has ready access to such advanced modalities. The present study suggests that widely used conventional sonography could help recognize the presence of liver injury in NAFLD patients in facilities without the equipment required to measure CAP or AC.

The precise relationship between severe fat deposition in the liver and development of liver injury remains unclear. One possible pathogenesis is that alterations in sinusoidal blood flow in enlarged liver due to severe fat deposition could activate Kupffer cells (hepatic macrophages), releasing inflammatory cytokines that then injure the liver[3)32)]. Another possibility is that an increased influx of fatty acids into the liver through portal flow derived from accumulated abdominal visceral adipose tissue, a primary factor in developing fatty liver together with obesity, could be metabolized to reactive oxygen species that injure the liver[1)33)]. Other inflammatory cytokines could be also released from this accumulated abdominal visceral adipose tissue, flow into the liver via the portal vein, and injure the liver[33)].

As a limitation, this study was a cross-sectional investigation, and follow-up observation is required to evaluate whether the presence or absence of liver injury could change according to changes in sonographic finding. Patients with liver injury were slightly younger than those without liver injury. Differences in dietary habits might also have somewhat affected the results of the present study. The subject cohort was limited to elderly women. Men or younger women might exhibit other characteristics. R2 was not completely measured within the scanning field in adults, since the measurement method adopted in the present study was that for children. This might be one reason why R1+R2 did not differ significantly between patients with and without liver injury (**Table 1**). The relationships between findings in the present study and values using CAP from VCTE or AC from ATI should be clarified.

V. Conclusion

The present study suggests that grade 3 fatty liver, hepatomegaly and/or deep attenuation could offer promising findings for easy, non-invasive prediction of the presence of liver injury in elderly women with NAFLD using conventional sonography.

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Disclosure

No potential conflicts of interest were disclosed.

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