	Ledipasvir-sofosbuvir for 12 weeks (n=171)	Ledipasvir-sofosbuvir plus ribavirin for 12 weeks (n=170)	Overall (n=341)
Age (years)	60 (9·2)	59 (9·5)	59 (9·4)
Patients aged ≥65 years	60 (35%)	52 (31%)	112 (33%)
Men	69 (40%)	73 (43%)	142 (42%)
Mean body-mass index (kg/m²)	23·3 (3·6)	23·3 (3·1)	22·9 (3·3)
Mean $\log_{10}$ HCV RNA (IU/mL)	6·6 (0·5)	6·6 (0·5)	6·6 (0·5)
Genotype			
1a	7 (4%)	4 (2%)	11 (3%)
1b	164 (96%)	166 (98%)	330 (97%)
IL28B alleles			
CC	86 (50%)	79 (46%)	165 (48%)
CT	78 (46%)	82 (48%)	160 (47%)
TT	7 (4%)	9 (5%)	16 (5%)
Cirrhosis			
No	130 (76%)	135 (79%)	265 (78%)
Yes	41 (24%)	35 (21%)	76 (22%)
Treatment history			
Treatment naive	83 (49%)	83 (49%)	166 (49%)
Previously treated	88 (51%)	87 (51%)	175 (51%)
Previous treatment regimen			
Peginterferon and ribavirin	54 (61%) of 88	47 (54%) of 87	101 (58%) of 175
Protease inhibitor, peginterferon, and ribavirin	17 (19%) of 88	23 (26%) of 87	40 (23%) of 175
Other	17 (19%) of 88	17 (20%) of 87	34 (19%) of 175
Response to previous treatment			
Non-responder	29 (33%) of 88	28 (32%) of 87	57 (33%) of 175
Breakthrough or relapse	44 (50%) of 88	44 (51%) of 87	88 (50%) of 175
Interferon intolerant	15 (17%) of 88	15 (17%) of 87	30 (17%) of 175

Data are mean (SD), n (%), or n/N (%) unless otherwise stated. HCV=hepatitis C virus.

Table 1: **Baseline characteristics**

score of 4 or an Ishak score of ≥5) or by Fibroscan score of more than 12·5 kPa. All patients were eligible to participate in a substudy to establish the steady-state pharmacokinetics of sofosbuvir (and its metabolite GS-331007), ledipasvir, and, where applicable, ribavirin.

Before enrolment and before any study procedures were undertaken, the study was approved by appropriate regulatory bodies and written informed consent was obtained from all patients. This study was done in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice. See appendix for protocol.

Randomisation and masking

Patients were randomly assigned (1:1) to receive either ledipasvir-sofosbuvir or ledipasvir-sofosbuvir and ribavirin. An interactive web response system was used to manage patient randomisation and treatment assignment. Randomisation of treatment-naive patients was stratified by the presence or absence of cirrhosis. Randomisation of previously treated patients was stratified by presence or absence of cirrhosis and by previous treatment category: relapser or breakthrough, non-responder, or interferon-intolerant. Within each strata, patients were sequentially assigned to treatments ledipasvir-sofosbuvir or ledipasvir-sofosbuvir plus ribavirin in a 1:1 ratio with a block size of 4. This study used an open-label design; both patients and study investigators were aware of treatment assignment.

Procedures

Patients in both treatment groups received 12 weeks of treatment with a fixed-dose combination tablet containing 90 mg of ledipasvir and 400 mg of sofosbuvir (ledipasvir-sofosbuvir) orally, once daily with or without food. When assigned, ribavirin was given orally as a divided, weight-based daily dose according to the Japanese Copegus product label (ie, patients ≤60 kg received 600 mg daily, patients >60 kg to ≤80 kg received 800 mg daily, and patients >80 kg received 1000 mg daily). After completion or early discontinuation of treatment, patients were followed up off-treatment for 24 weeks.

Serum hepatitis C virus RNA concentrations were measured with the COBAS TaqMan hepatitis C virus Test, version 2·0 for use with the High Pure System (Roche, IN, USA), with a lower limit of quantification for hepatitis C virus RNA of less than 25 IU/mL. Virological breakthrough was defined as the presence during treatment of hepatitis C virus RNA of at least 25 IU/mL in a patient with previous documentation of on-treatment concentrations of hepatitis C virus RNA of less than 25 IU/mL. Relapse was defined as the confirmed presence of hepatitis C virus RNA of at least 25 IU/mL at any time during the post-treatment follow-up after documented hepatitis C virus RNA of less than 25 IU/mL at the end of treatment. For all patients, the *IL28B* genotype was established by PCR amplification and sequencing of the rs12979860 single nucleotide polymorphism.

Deep sequencing of the NS5A and NS5B regions of the hepatitis C virus was done for all patients at baseline. For all patients who had virological failure (defined as virological breakthrough, relapse, rebound [>1 $\log_{10}$ IU/mL increase in hepatitis C virus RNA from nadir while on treatment with two consecutive values, or last available on-treatment measurement with no subsequent follow up values], or non-response [hepatitis C virus RNA persistently >25 IU/mL through 8 weeks of treatment]), deep sequencing of the NS5A and NS5B regions was done at both baseline and at the time of virological failure. The resulting sequences were compared with sequences from baseline samples to detect treatment emergent resistance-associated variants. Resistance-associated variants present at greater than 1% of sequence reads were regarded as significant.

The population pharmacokinetic variables for ledipasvir, sofosbuvir, and GS-331007 (the predominant circulating metabolite of sofosbuvir) were computed

for all patients from available concentration data from intensive or sparse samples with previously established population pharmacokinetic models.

Outcomes

The primary efficacy endpoint of the study was the proportion of patients who achieved SVR12. Analysis of this endpoint included all patients who were randomly assigned and received at least one dose of study drug. SVR12 is the proportion of patients with hepatitis C virus RNA less than LLOQ (ie <25 IU/mL) 12 weeks after completion of treatment. Secondary outcomes were to establish the proportion of patients who attained sustained virological response at 4 and 24 weeks after discontinuation of therapy (SVR4 and SVR24), to assess the kinetics of circulating HCV RNA during treatment and after treatment discontinuation, and to assess the emergence of viral resistance to sofosbuvir and ledipasvir during treatment and after treatment discontinuation. Safety was assessed in all patients by physical examination and by review of adverse events and blood and urine samples for clinical laboratory testing. Reduction or discontinuation of ribavirin dosing because of toxic effects was done according to the Japanese Copegus product label. Patients were not permitted to use erythropoiesis-stimulating drugs, granulocyte colony-stimulating factor, or thrombopoietin mimetics from 28 days before screening to the end of treatment.

Statistical analysis

We calculated the SVR12 for each treatment group along with the two-sided 95% CI using the exact binomial distribution (the Clopper-Pearson method). For treatment-naive patients without cirrhosis, the SVR12 rate was compared with the adjusted historical sustained virological response null rate of 63% using a two-sided exact one-sample binomial test. The appendix shows the method of calculating the historical sustained virological response control rate. No statistical hypothesis testing was done for outcomes in treatment-naive patients with cirrhosis or in previously treated patients.

We estimated that a sample size of 45 treatment-naive patients without cirrhosis will provide at least 90% power to detect a 23% improvement in SVR12 rate from the adjusted historical control rate of 63% using a two-sided exact one-sample binomial test at a significance level of 0·025 based on a Bonferroni correction. The targeted enrolment for the pharmacokinetics substudy was roughly 15 each of treatment-naive and previously treated patients. SAS Software version 9.2 was used for statistical analysis (SAS Institute, NC, USA).

This trial is registered with ClinicalTrials.gov, number NCT01975675.

Role of the funding source

The funder of the study oversaw trial management, data collection, statistical analyses, and the writing and review of the report. The corresponding author had full access to all data in the study and had final responsibility for the decision to submit for publication.

	Ledipasvir-sofosbuvir for 12 weeks (n=171)	Ledipasvir-sofosbuvir plus ribavirin for 12 weeks (n=170)
Treatment week 2	**136 (80%) of 171**	**136 (80%) of 169**
Treatment week 4	171 (100%) of 171	169 (100%) of 169
End of treatment	**171 (100%) of 171**	**168 (100%) of 168**
SVR4	171 (100%)	167 (98%)
SVR12	**171 (100%; 98–100)**	**167 (98%; 95–100)**
Virological failure		
During treatment	0	0
Relapse	0	1 (<1%)
Imputed	0	2* (1%)

Data are n/N (%), n (%), or n (%, 95% CI). SVR4=sustained virological response at week 4 after treatment. SVR12=sustained virological response at week 12 after treatment. *One patient discontinued from the study on day 6 because of adverse events; one patient discontinued from the study on day 62 because of adverse events and died the next day.

Table 2: **Response during and after treatment**

Results

Screening for the trial began on Oct 15, 2013, with the last patient enrolled on Dec 13, 2013. 421 patients were screened, of whom all 341 were randomly assigned and received study treatment (figure; appendix). Overall, most patients (58%) were female and almost all patients (97%) had genotype 1b hepatitis C virus (table 1). The mean age was 59 years, and 112 (33%) patients were aged 65 years or older. 76 patients (22%) had compensated cirrhosis at baseline. Overall, 76 (22%) patients had hepatitis C virus NS5A resistance-associated variants at baseline, 42 (25%) of the 171 receiving ledipasvir-sofosbuvir and 34 (20%) of the 170 receiving ledipasvir-sofosbuvir plus ribavirin. Of the 175 previously treated patients, 40 (23%) had received previous treatment with a triple therapy regimen consisting of pegylated interferon alfa and ribavirin plus a protease inhibitor, such as telaprevir (14), simeprevir (13), vaniprevir (eight), or faldaprevir (five). 88 (50%) of the previously treated patients had relapse or breakthrough during previous hepatitis C virus treatment, 57 (33%) had no response, and 30 (17%) were interferon intolerant.

Overall, 338 (99%) of 341 patients achieved SVR12, including all 171 patients (100%) receiving ledipasvir-sofosbuvir (95% CI 98–100) and 167 (98%) of the 170 patients receiving ledipasvir-sofosbuvir plus ribavirin (95% CI 95–100; table 2). In treatment-naive patients without cirrhosis, 70 (100%) of 70 patients receiving ledipasvir-sofosbuvir and 69 (97%) of 71 patients receiving ledipasvir-sofosbuvir plus ribavirin achieved SVR12, thereby meeting the primary efficacy endpoint of SVR12 better than the prespecified adjusted historical sustained virological response null rate of 63% ($p<0·0001$ for both comparisons). There was 100% concordance between

SVR4, SVR12, and SVR24. Adherence to study drugs was high in both groups (appendix).

Response in patient subgroups is shown in the appendix. All 175 (100%) patients previously treated achieved SVR12, including all 40 who did not respond to previous treatment with a protease inhibitor-based regimen. In patients with NS5A resistance-associated variants at baseline, 42 (100%) of 42 of those receiving ledipasvir-sofosbuvir and 33 (97%) of 34 receiving ledipasvir-sofosbuvir plus ribavirin achieved SVR12.

In view of the uniformly high levels of SVR12 in all groups, identification of factors predictive of virological success or failure was not possible. Patients with factors that have historically been associated with lower response—non-response to previous treatment, the presence of cirrhosis, non-CC *IL28B* genotype—had SVR12 rates similar to patients without unfavourable characteristics. The presence of ribavirin in the regimen was not associated with increased SVR12.

In 341 patients enrolled and treated, one had virological failure. This patient, a treatment-naive 55-year-old woman without cirrhosis who was receiving ledipasvir-sofosbuvir plus ribavirin, relapsed by post-treatment week 4 after completion of treatment. This patient had adherence rates of more than 99% for both ledipasvir-sofosbuvir and ribavirin (800 mg daily) based on pill counts from returned study medication.

The patient who had a viral relapse had genotype 1b hepatitis C virus infection and the Y93H (>99%) NS5A resistance-associated variants at baseline and at post-treatment week 4. No other NS5A resistance-associated variants were detected at post-treatment week 4. No NS5B nucleoside inhibitor variants and no treatment-emergent variants were detected in any patient at any timepoint tested.

The mean (coefficient of variation [CV], %) of steady-state for ledipasvir for AUC_{0-24} (defined as area under the concentration-time curve from 0 h to 24 h) was 11 500 (56·0) ng×h per mL, for C_{max} (defined as maximum or peak plasma concentration) was 485 (48·3) ng/mL, and for C_{tau} (the plasma concentration at the end of the dosing interval) was 326 (55·9) ng/mL. The mean (CV, %) was 1560 (43·1) ng×h per mL for steady-state AUC_{0-24} and 579 (40·3) ng/mL for C_{max} for sofosbuvir (n=67). For its metabolite GS-331007 (n=341), steady-state AUC_{0-24} was 12 600 (23·9) ng×h per mL, and C_{max} was 723 (22·1) ng/mL. We noted no clinically relevant differences in the pharmacokinetics of ledipasvir, sofosbuvir, or GS-331007 on the basis of creatinine clearance, age, sex, body-mass index, cirrhosis status, previous treatment experience, or ribavirin treatment regimen.

Overall, 240 (70%) of 341 patients had at least one treatment-emergent adverse event. Of the patients who had adverse events, most (201 [84%] of 240) had only mild (grade 1) events. The most common adverse events were nasopharyngitis (common cold), anaemia, and headache. Adverse events were higher in patients receiving ledipasvir-sofosbuvir plus ribavirin (128 [75%] of 170) than in those receiving ledipasvir-sofosbuvir only (112 [65%] of 171). Patients receiving ribavirin had higher rates of events known to be associated with this drug—anaemia, pruritus, rash, and nausea. Modification of ribavirin dose was needed in 20 (12%) of 170 patients (appendix), mainly because of anaemia or decreased haemoglobin. Two patients receiving ledipasvir-sofosbuvir plus ribavirin discontinued all study treatment prematurely because of adverse events: one patient discontinued treatment because of a ribavirin-associated morbilliform drug eruption on day 6 of treatment, and one patient had a cardiac arrest leading to death 1 day after stopping treatment. None of the patients randomly assigned to receive ledipasvir-sofosbuvir discontinued the study drugs because of adverse events.

Five patients had a treatment-emergent serious adverse event, of which two were judged to be related to study treatment. One patient, a 67-year-old, treatment-naive man with a medical history including liver cirrhosis, diabetes mellitus, sarcoidosis, pulmonary fibrosis, splenectomy, and chronic gastritis was randomly assigned to receive ledipasvir-sofosbuvir plus ribavirin. On day 62 (week 8) of treatment, the patient had nausea, vomiting, diarrhoea, and fever suggestive of a serious underlying infection; at that time, study treatment was discontinued. The next day, he had a cardiac arrest and died. The investigator assessed the death as related to study treatment, but also listed possible alternate causes, including pre-existing conditions, persisting infection, and other, unspecified, drugs. The investigator commentary reported a low likelihood of association between death and the investigational drugs, with the most probable explanation being viral gastrointestinal infection leading to cardiac arrest.

The other serious adverse event judged by an investigator as related to study treatment was an acute myocardial infarction that occurred in a treatment-experienced, 71-year old man, who had previously received pegylated interferon alfa-2b and ribavirin and had a history of liver cirrhosis and hypertension. This patient received 12 weeks of ledipasvir-sofosbuvir plus ribavirin. 9 days after completion of treatment, the patient had an acute myocardial infarction. The patient underwent cardiac catheterisation and was identified to have complete stenosis of the left anterior descending artery and 99% stenosis of the right coronary artery, which probably represented pre-existing chronic conditions (appendix).

The rate of adverse events in patients younger than 65 years (155 [68%] of 229) was lower than that identified in patients aged 65 years and older (85 [76%] of 112). Overall, patients with cirrhosis did not have a substantially higher rate of adverse events than did patients without cirrhosis (appendix).

12 patients (7%) receiving ledipasvir-sofosbuvir and 14 (8%) receiving ledipasvir-sofosbuvir plus ribavirin had

	Ledipasvir-sofosbuvir for 12 weeks (n=171)	Ledipasvir-sofosbuvir plus ribavirin for 12 weeks (n=170)
Any adverse event	112 (65%)	128 (75%)
Discontinuations because of adverse events	0	2 (1%)
Deaths	0	1 (<1%)
Serious adverse events	3 (2%)	2 (1%)
Acute myocardial infarction	0	1 (<1%)
Cardiac arrest	0	1 (<1%)
Hepatocellular carcinoma	1 (<1%)	0
Oesophageal varices haemorrhage	1 (<1%)	0
Wrist fracture	1 (<1%)	0
Common adverse events*		
Nasopharyngitis	50 (29%)	40 (24%)
Anaemia	3 (2%)	23 (14%)
Headache	12 (7%)	15 (9%)
Pruritus	6 (4%)	13 (8%)
Rash	5 (3%)	14 (8%)
Malaise	9 (5%)	9 (5%)
Stomatitis	6 (4%)	10 (6%)
Nausea	5 (3%)	9 (5%)
Haematological abnormality		
Decreased haemoglobin		
<100 g/L	4 (2%)	10 (6%)
<85 g/L	1 (<1%)	0
Lymphocyte count $0{\cdot}35\times10^9$ to $<0{\cdot}50\times10^9$ per L	3 (2%)	1 (<1%)
Neutrophil count $0{\cdot}50\times10^9$ to $<0{\cdot}75\times10^9$ per L	2 (1%)	0
Platelet count 25×10^9 to $<50\times10^9$ per L	1 (<1%)	0

Data are n (%). *Adverse events occurring in at least 5% of patients in any group.

Table 3: **Safety**

grade 3 laboratory abnormalities and none of the patients had any grade 4 abnormalities (appendix). The only clinically significant difference in the prevalence of these laboratory abnormalities between the treatment groups was that a greater number of patients in the ribavirin-treated group had grade 3 reductions in haemoglobin than patients receiving ledipasvir-sofosbuvir only (five [3%] of 170 *vs* two [1%] of 171). For patients receiving ribavirin, the mean change in haemoglobin from baseline to the end of treatment week 12 was −16 g/L (SD 11·5) versus −3 g/L (SD 8·2) for those receiving ledipasvir-sofosbuvir only. In patients receiving ledipasvir-sofosbuvir and ribavirin, ten (6%) had at least one post-baseline haemoglobin value lower than 100 g/L and none had a post-baseline value lower than 85 g/L versus four (2%) who had at least one post-baseline haemoglobin value lower than 100 g/L and one (≤1%) who had a post-baseline value lower than 85 g/L in the group receiving ledipasvir-sofosbuvir only.

Discussion

In this trial, 12 weeks of treatment with the fixed-dose combination of ledipasvir and sofosbuvir without ribavirin was well tolerated and resulted in SVR12 in all 171 patients (100%) treated, including patients typically difficult to treat, including those with cirrhosis, or baseline NS5A resistant variants, and those who had previously not responded well to other hepatitis C virus treatment regimens, including protease inhibitor-based therapies. In the ledipasvir-sofosbuvir plus ribavirin group, 167 (98%) of 170 patients achieved SVR12. The addition of ribavirin to ledipasvir-sofosbuvir did not increase the number of patients who achieved SVR12, but did increase the number of patients who had adverse events, even though ribavirin dosing was lower in this study, in accordance with Copegus dosing recommendations in Japan, than in other studies. These findings are broadly in keeping with results from the phase 3 registrational trials of ledipasvir-sofosbuvir in patients with genotype 1 hepatitis C virus done in the USA and Europe.[13–15] In those trials, 12 weeks of ledipasvir-sofosbuvir resulted in 99% of treatment-naive patients and 94% previously treated patients achieving SVR12. Similarly to the present trial, patients receiving ribavirin had similar rates of sustained virological response, but increased rates of adverse events. A previous phase 1 study[16] showed that no clinically significant differences existed in the pharmacokinetics of sofosbuvir, GS-331007, or ledipasvir between Japanese and white patients.

Our findings suggest that many of the host and viral factors that are predictive of outcomes with interferon-based treatments—previous treatment response, hepatitis C virus genotype (1a *vs* 1b), age, baseline viral load, *IL28B* genotype, cirrhosis status, presence of baseline resistant variants, ribavirin exposure, and early treatment response—are not of clinical relevance in the use of the ledipasvir-sofosbuvir fixed-dose combination. Although most patients in this study were hepatitis C virus genotype 1b, consistent with genotype distribution in Japan,[1] the efficacy of ledipasvir-sofosbuvir in hepatitis C virus genotype 1a has been extensively examined in overseas studies and no clinically important differences in response have been identified between subtypes.[13–15] This uniformity of response to the regimen, and its potential for use in patients with contraindications to or intolerance of interferon or ribavirin, suggests that this combination might have broad applications.

Ledipasvir-sofosbuvir with and without ribavirin seemed to be well tolerated by patients with hepatitis C virus infection, with adverse events known to be associated with ribavirin therapy.[17] Two serious cardiac adverse events (arrest and myocardial infarction) occurred, which were thought to be possibly related to study drug by the investigator; however, in both cases, alternative causes for these adverse events were also proposed by the investigator as likely contributory (infection and pre-existing disease). Data from the present trial and those previously reported

for the ledipasvir-sofosbuvir development programme do not suggest that ledipasvir-sofosbuvir confers any increased risk of cardiac events.

With the exception of nasopharyngitis, the type, frequency, and severity of the adverse events reported in patients receiving ledipasvir-sofosbuvir were similar to those seen in untreated patients from the control group of a phase 3 trial.[18] No clinically relevant differences in pharmacokinetic variables between Japanese patients in this trial and the population in large phase 3 trials exist.

The proportion of patients with nasopharyngitis was much higher in this study (22–28%; table 3) than in large phase 3 trials, in which the proportion of patients with nasopharyngitis ranged from 1% or less to 9%. The increased rate of nasopharyngitis seen in this study is perhaps attributable to the fact that this study was done during winter in Japan and the population enrolled in the trial.

Limitations of this study include the open-label design and absence of an active comparator. The trial design could introduce bias in reported adverse events and efficacy results secondary to influence on adherence and tolerability (eg, if patients know that they are assigned to receive ribavirin, whereas others in the study are not, they might be selectively non-adherent to ribavirin or be more likely to report known side-effects of ribavirin). The absence of an active comparator also precluded us from assessing which adverse events resulted from ledipasvir-sofosbuvir.

Our findings suggest that the all-oral, interferon-free and ribavirin-free fixed-dose combination of ledipasvir and sofosbuvir given as one tablet once daily might be an important advancement in the treatment of hepatitis C virus genotype 1 in Japan.

Declaration of interests

MM has received speakers fees from Abbott Japan Co, Astellas Pharma, Bristol-Myers Squibb, Chugai Pharmaceutical Co, Daiichi Sankyo Co, Dainippon Sumitomo Pharma Co, Eisai Co, G&G Co, GlaxoSmithKline, Minophagen Pharmaceutical Co, Merck Sharp & Dohme, Otsuka Pharmaceutical Co, Taisho Toyama Pharmaceutical Co, Takeda Pharmaceutical Co, The Chemo-Sero-Therapeutic Research Institute, Toray Industries, and Sysmex Corporation; and received research grants from Asahi Kasei Pharma Corporation, Mitsubishi Tanabe Pharma Corp, G&G Science Co, SRL Technical Services, and received travel grants from G&G Science Co. OY has received speakers fees from Merck Sharp & Dohme, Kowa Souku, Sysmex, Chugai Pharmaceutical Co, GlaxoSmithKline, Bristol-Myers Squibb, Ajinomoto-Seiyaku, Bayer, Abbott, Given Imaging, Mitsubishi Tanabe Pharm, Taiko Yakuhin, Dainippon Sumitomo Pharm, and Igaku-Seibutsugaku Institute. NS has received speakers' fees and research grants from Merck Sharp & Dohme, Bristol-Myers Squibb, Janssen Pharmaceuticals, Otsuka Pharmaceutical, Dainippon Sumitomo Pharma, Chugai Pharmaceutical Co, Mitsubishi Tanabe Pharma, Gilead Sciences, Takeda Pharmaceutical, Daiichi Sankyo Co, and Ajinomoto Pharmaceuticals. MY has received speakers fees from Otsuka Pharmaceutical Co. HT has received speakers fees from Chugai Pharmaceutical Co, Eisai, Bayer Co, Merck Sharp & Dohme, Nippon Kayaku, Boehringer Ingelheim, Novartis Pharma, Otsuka Pharmaceutical, and Ajimoto. TU has received research funds from Merck Sharp & Dohme. HY has received speakers' fees from Chugai Pharmaceutical Co, Merck Sharp & Dohme, GlaxoSmithKline, Bristol-Myers Squibb, and Otsuka, and has received research funds from Chugai Pharmaceutical Co. TI has received speakers' fees from Merck Sharp & Dohme, Chugai Pharmaceutical Co, Janssen Pharmaceutical, Daiichi Sankyo Company, Bristol-Myers Squibb, Mitsubishi Tanabe Pharma Corporation, and Sumitomo Dainippon Pharma. NT has received speakers' fees and been an adviser to Novartis Pharma, Shianogi Pharma, AstraZeneca, Kyorin Pharma, and Zeria Pharma. KNi has received speakers' fees from Ajinomoto Pharmaceuticals Co, Janssen Pharmaceuticals, and Merck Sharp & Dohme. YU has received speakers' fees from Bristol-Myers Squibb, Janssen Japan, and Tanabe Mitshubishi Pharma. NI has received speakers fees and served as an adviser to Merck Sharp & Dohme, Chugai Pharmaceutical Co, Daiichi Sankyo Co, Bayer Co, Bristol-Myers Squibb Co, Boehringer Ingelheim Co, Janssen Co, Gilead Sciences, Shionogi Co, Kowa Co, Eisai Co, Taiko Co, Totsuka Co, and Ajinomoto Co. MOmota has received fees for being a speaker, consultant, and advisory board member for Bayer Co, Boehringer Ingelheim, Bristol-Myers Squibb, Otsuka, Astellas, Gilead Sciences, Chugai, Mitsubishi Tanabe, Kyorin, Merck Sharp & Dohme, Dainippon Sumitomo, Vertex Pharmaceuticals, Takeda, and Zeria. JB, BG, AI, MOmote, HM, KG, PSP, SJK, WTS, and JGM are employees of and own stock in Gilead Sciences. All other authors declare no competing interests.

Acknowledgments

This study was sponsored by Gilead Sciences. Writing assistance was provided by David McNeel and Shampa De-Oertel of Gilead Sciences.

References

1 Chung H, Ueda T, Kudo M. Changing trends in hepatitis C infection over the past 50 years in Japan. *Intervirology* 2010; **53:** 39–43.

2 Drafting Committee for Hepatitis Management Guidelines, the Japan Society of Hepatology. JSH Guidelines for the Management of Hepatitis C Virus Infection: a 2014 update for genotype 1. *Hepatol Res* 2014; **44** (suppl 1): 59–70.

3 Hayashi N, Izumi N, Kumada H, et al. Simeprevir with peginterferon/ribavirin for treatment-naïve hepatitis C genotype 1 patients in Japan: CONCERTO-1, a phase III trial. *J Hepatol* 2014; **61:** 219–27.

4 Izumi N, Hayashi N, Kumada H, et al. Once-daily simeprevir with peginterferon and ribavirin for treatment-experienced HCV genotype 1-infected patients in Japan: the CONCERTO-2 and CONCERTO-3 studies. *J Gastroenterol* 2014; **49:** 941–53.

5 Kumada H, Hayashi N, Izumi N, et al. Simeprevir (TMC435) once daily with peginterferon-α-2b and ribavirin in patients with genotype 1 hepatitis C virus infection: The CONCERTO-4 study. *Hepatol Res* 2014; published online June 24. DOI:10.1111/hepr.12375.

6 Zeuzem S, Berg T, Gane E, et al. Simeprevir increases rate of sustained virologic response among treatment-experienced patients with HCV genotype-1 infection: a phase IIb trial. *Gastroenterology* 2014; **146:** 430–41.

7 Gane E. Hepatitis C beware-the end is nigh. *Lancet* 2014; **384:** 1557–60.

8 Kumada H, Suzuki Y, Ikeda K, et al. Daclatasvir plus asunaprevir for chronic HCV genotype 1b infection. *Hepatology* 2014; **59:** 2083–91.

9 Daklinza (daclatasvir) tablets: Japanese prescribing information. July, 2014. http://www.kodc.or.kr/home/drug_info/readDownloadFile.do;jsessionid=C5A2DAD4B6EE55414F79E40385C244B1?kdcode=Z40000418&drugCd=B003301&type=eng (accessed Jan 28, 2015). Tokyo: Bristol-Myers Squibb.

10 Sunvepra (asunaprevir) capsules: Japanese prescribing information. July, 2014. http://www.kodc.or.kr/home/drug_info/readDownloadFile.do;jsessionid=26C94468C2AEC9926592D5D1A6B96767?kdcode=Z40000419&drugCd=B003311&type=eng (accessed Jan 28, 2015).

11 Sovaldi (sofosbuvir) tablets: US prescribing information. December, 2013. http://www.gilead.com/~/media/Files/pdfs/medicines/liver-disease/sovaldi/sovaldi_pi.pdf (accessed Jan 28, 2015). Foster City: Gilead Sciences.

12 Lawitz EJ, Gruener D, Hill JM, et al. A phase 1, randomized, placebo-controlled, 3-day, dose-ranging study of GS-5885, an NS5A inhibitor, in patients with genotype 1 hepatitis C. *J Hepatol* 2012; **57:** 24–31.

13 Afdhal N, Zeuzem S, Kwo P, et al. Ledipasvir and sofosbuvir for untreated HCV genotype 1 infection. *N Engl J Med* 2014; **370:** 1889–98.

14 Afdhal N, Reddy KR, Nelson DR, et al. Ledipasvir and sofosbuvir for previously treated HCV genotype 1 infection. *N Engl J Med* 2014; **370**: 1483–93.

15 Kowdley KV, Gordon SC, Reddy KR, et al. Ledipasvir and sofosbuvir for 8 or 12 weeks for chronic HCV without cirrhosis. *N Engl J Med* 2014; **370**: 1879–88.

16 Kirby B, German P, Shen G, et al. No ethnic differences in the pharmacokinetics of sofosbuvir or sofosbuvir/ledipasvir fixed dose combination in Japanese and Caucasian subjects. *Hepatol Res* 2013; 7 (suppl): S375–76.

17 Rebetol (ribavirin) capsules: US prescribing information. NJ: Merck Sharp & Dohme, 2003.

18 Jacobson IM, Gordon SC, Kowdley KV, et al. Sofosbuvir for hepatitis C genotype 2 or 3 in patients without treatment options. *N Engl J Med* 2013; **368**: 1867–77.

Obesity Facts
The European Journal of Obesity

Obes Facts 2015;8:1–16
DOI: 10.1159/000373881
Received: January 29, 2014
Accepted: July 31, 2014
Published online: January 29, 2015

1662–4033/15/0081–0001$39.50/0
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Original Article

Association of Peripheral Total and Differential Leukocyte Counts with Obesity-Related Complications in Young Adults

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Key Words
Inflammation · Obesity · Insulin resistance · Dyslipidemia · Body fat

Abstract
Objective: Obesity has been demonstrated to be associated with elevated leukocytes in adults and children. This study assessed the associations between peripheral total and differential leukocyte counts and obesity-related complications in young adults. ***Methods:*** 12 obese (median age 21.5 (range 19–28) years, median BMI 35.7 (range 32.0–44.9) kg/m^2) and 11 normal (median age 23 (range 18–27) years, median BMI 19.5 (range 18.1–21.7) kg/m^2) adults were enrolled. Complete blood count and serum levels of liver enzymes, fasting blood glucose, insulin and lipids were measured, and the homeostasis model assessment of insulin resistance was calculated. Fat mass was calculated using a bioimpedance analysis device, and ultrasonography was performed to measure fat thickness and to detect fatty change of the liver. ***Results:*** Total leukocyte and monocyte counts were significantly increased in obese young adults. Total leukocyte count was associated with liver enzyme levels, insulin resistance as well as visceral and subcutaneous fat thickness. Neutrophil count was associated with insulin resistance. Lymphocyte count was associated with serum liver enzymes, insulin resistance, and dyslipidemia. Monocyte count was associated with serum liver enzyme, insulin resistance, visceral and subcutaneous fat thickness, body fat mass, and percentage body fat. ***Conclusion:*** The results of this study suggest that chronic low-grade systemic inflammation is associated with obesity-related complications such as nonalcoholic fatty liver disease, insulin resistance, and dyslipidemia in young adults.

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KARGER 125 YEARS

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DOI: 10.1159/000373881

Introduction

Obesity is one of the leading causes of morbidity and mortality in both adults and children, and its prevalence is increasing in the younger population [1, 2]. Because obesity is associated with type 2 diabetes, hypertension, coronary heart disease, and certain forms of cancer, early prevention of obesity has assumed great importance. Recently, it has become clear that obesity is also associated with immunological abnormalities [3]. Inflammatory cells such as lymphocytes and macrophages have been shown to infiltrate into the adipose tissue in obese humans, and proinflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) are produced by those inflammatory cells and adipocytes, causing chronic low-grade systemic inflammation [2, 4, 5].

Studies in the last few decades have demonstrated that obese adults and children have elevated total leukocytes [6–14], the majority of which appear to be related to monocytes [15]; however, other types of leukocytes – such as lymphocytes and neutrophils – may also be elevated [9]. It is not known whether the number of these inflammatory cells in circulation is associated with obesity-related complications such as nonalcoholic fatty liver disease (NAFLD), insulin resistance, and dyslipidemia in young adults.

We therefore measured serum liver enzyme and lipid levels, insulin resistance, and fat volume and investigated the association of peripheral total and differential leukocyte counts with obesity-related complications in young adults.

Material and Methods

Subjects

12 students, each with a BMI of ≥35 kg/m^2 (obese group), were recruited from the participants at the 2013 annual health check-up held at the Health Care Center, Hokkaido University, Sapporo, Japan. In addition, 11 age-matched students, each with a BMI of 20–22 kg/m^2 (control group), were recruited. None of the subjects were on any medications, none were smokers, and all were negative for hepatitis B surface antigen, anti-hepatitis C virus antibody and anti-nuclear antigen, suggestive that they are free from chronic viral hepatitis and autoimmune hepatitis. Their alcohol ingestion was <140 g/week. This study was approved by the Ethics Committee of Hokkaido University Graduate School of Medicine. After the purpose of the study had been explained to all subjects, written informed consent was obtained according to the tenets of the 1975 Declaration of Helsinki.

Blood Examination

Blood samples were collected into ethylene diamine tetraacetic acid-dipotassium salt dihydrate (EDTA-2K) tubes, sodium fluoride (NaF) tubes, and in plain polystyrene tubes after overnight fasting. Complete blood count and the subtype fractions of leukocytes were analyzed by an automated blood cell counter (XS-1000i; Sysmex, Kobe, Japan). Other biochemical measurements were performed at SRL Inc. (Tokyo, Japan), and the following data were collected: total bilirubin (T-Bil, vanadate oxidase method, CV% = 1.351), aspartate aminotransferase (AST, standardization-adjusted ultraviolet method, CV% = 1.262), alanine aminotransferase (ALT, standardization-adjusted ultraviolet method, CV% = 1.309), γ-glutamyl transpeptidase (γ-GTP, L-glutamyl-3-carboxy-4-nitroanilide substrate method, CV% = 1.895), cholinesterase (ChE, Japan Society of Clinical Chemistry (JSCC) method, CV% = 0.791), fasting blood glucose (FBG, hexokinase method, CV% = 1.12), hemoglobin A1c (HbA1c, latex agglutination inhibition method, CV% = 2.33), insulin (chemiluminescent enzyme immunoassay, CV% = 4.25), total cholesterol (T-CHO, cholesterol dehydrogenase-ultraviolet method, CV% = 1.144), high-density lipoprotein cholesterol (HDL-C, selective inhibition method, CV% = 1.571), low-density lipoprotein cholesterol (LDL-C, direct method, CV = 2.504), triglyceride (TG, glycerol kinase-glycerol-3-phosphate oxidase-peroxidase method, CV% = 1.134), total protein (TP, Biuret method, CV% = 1.622), albumin (Alb, modified bromocresol purple method, CV% = 1.348), amylase (AMY, JSCC method, CV% = 1.174), blood urea nitrogen (BUN, urease method, CV% = 1.173), creatinine (Cre, enzymatic method, CV% = 1.304), uric acid (UA, uricase-peroxidase method, CV% = 1.253),

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and C-reactive protein (CRP, latex immunonephelometric method, CV% = 1.821). All tests were performed on the day of blood collection. The insulin resistance index was calculated from the homeostasis model assessment of insulin resistance (HOMA-IR) using the following equation: (fasting insulin in μU/l) × (FBG in mmol/l) / 22.5 [16].

Body Composition Measurement

Body composition, including body fat mass and percentage body fat, was calculated using a bioimpedance analysis device (InBody S20; Biospace, Seoul, South Korea), which appears to be noninvasive and accurate for evaluating body composition [17].

Ultrasonography

Ultrasonography was performed for all subjects in the supine position using a probe located 1 cm above the umbilical scar on the abdominal midline (LOGIQ S7, GE Healthcare). Subcutaneous fat thickness was measured as the distance (cm) between the skin and the external surface of the rectus abdominis muscle, and visceral fat thickness was measured as the distance between the internal surface of the rectus abdominis muscle and the anterior wall of the aorta [18]. Fatty liver was defined on the basis of a combination of liver-kidney contrast (bright liver) and vascular blurring [19].

Statistical Analyses

Data are expressed as median with range. Differences in each parameter between the obese and control groups were examined for statistical significance using a nonparametric Mann-Whitney test. Spearman's correlation coefficient and linear regression were used to assess the simple relationship between the variables. The significance threshold was set at $p < 0.05$.

Results

The characteristics of 11 control and 12 obese subjects are shown in table 1. Both groups had a similar age distribution ($p = 0.126$). The obese group included only males, whereas 4 out of 11 subjects in the control group were female. Fatty change of the liver was not observed in the control group, whereas 9 out of 12 subjects in the obese group exhibited fatty change of the liver on ultrasonography and were diagnosed with NAFLD. Body composition measurement using a bioimpedance analysis device demonstrated that body fat mass and percentage body fat were significantly higher in the obese group than in the control group. Furthermore, visceral fat thickness and subcutaneous fat thickness as measured by ultrasonography were significantly increased in the obese group compared with those in the control group. The obese group exhibited significantly higher serum levels of liver enzymes such as AST, ALT, γ-GTP, and ChE. Serum insulin, HOMA-IR, LDL-C, TG, UA, and CRP levels were significantly higher in the obese group than in the control group. On the other hand, Alb, AMY, and HDL-C levels were significantly higher in the control group than in the obese group. FBG, HbA1c, T-Bil, T-CHO, TP, BUN, and Cre levels were comparable between groups.

The complete blood count of the subjects is presented in table 2. The peripheral erythrocyte and total leukocyte counts were significantly higher in the obese group than in the control group. Particularly, significant increases were noted in the monocyte count in the obese group compared with those in the control group. There was no significant difference in peripheral neutrophil, lymphocyte as well as eosinophil and basophil counts between groups.

We then analyzed whether peripheral total leukocyte, neutrophil, lymphocyte, and monocyte counts correlated with the serum levels of liver enzymes in all 23 subjects. A significant positive correlation was found between total leukocyte, lymphocyte or monocyte counts and AST, ALT, γ-GTP, and ChE levels (fig. 1).

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Table 1. Subject characteristics and biochemical data

	Control group (n = 11)	Obese group (n = 12)
Male/female	7/4	12/0
Age, years	23 (18–27)	21.5 (19–28)
BMI, kg/m^2	19.5 (18.1–21.7)	35.7 (32.0–44.9)***
BFM, kg	8.2 (6.0–14.9)	41.8 (26.6–63.0)***
PBF, %	14.6 (10.6–29.8)	39.1 (26.4–48.6)***
Visceral fat thickness, mm	23.2 (17.2–47.3)	69.4 (39.8–92.1)***
Subcutaneous fat thickness, mm	5.4 (3.2–9.9)	15.8 (11.5–29.8)***
Existence of fatty liver (+/–)	0/11	9/3
T-Bil, μmol/l	15.4 (5.1–34.2)	13.7 (6.8–35.9)
AST, U/l	17 (14–35)	25 (15–89)**
ALT, U/l	12 (9–42)	39.5 (13–189)***
γ-GTP, U/l	17 (11–28)	33.5 (14–133)**
ChE, U/l	276 (196–345)	406 (306–479)***
FBG, mmol/l	4.6 (4.3–5.1)	4.8 (4.2–5.3)
HbA1c, %	5.0 (4.7–5.9)	5.1 (4.5–5.6)
Insulin, μIU/ml	3.1 (2.2–6.4)	12.1 (6.3–32.4)***
HOMA-IR	0.7 (0.4–1.5)	2.6 (1.5–6.9)***
T-CHO, mmol/l	4.3 (3.7–5.5)	4.9 (3.6–10.5)
HDL-C, mmol/l	1.6 (1.3–2.5)	1.1 (0.8–1.3)***
LDL-C, mmol/l	2.2 (1.2–3.5)	3.4 (2.3–8.1)**
TG, mmol/l	0.5 (0.4–1.7)	1.2 (0.5–3.6)**
TP, g/l	73 (69–82)	73 (68–79)
Alb, g/l	47 (41–49)	44 (40–48) *
AMY, U/l	74 (59–229)	57.5 (36–75) **
BUN, mmol/l	4.2 (3.3–5.7)	3.8 (2.7–5.2)
Cre, μmol/l	71.6 (47.7–84.9)	67.6 (60.1–91.1)
UA, μmol/l	350.9 (148.7–452.0)	443.1 (232.0–535.3)**
CRP, mg/l	0.2 (0.0–4.8)	1.6 (0.2–28.1)**

BFM = Body fat mass; PBF = percent body fat; T-Bil = total bilirubin; AST = aspartate aminotransferase; ALT = alanine aminotransferase; γ-GTP = γ-glutamyl transpeptidase; ChE = cholinesterase; FBG = fasting blood glucose; HbA1c = hemoglobin A1c; HOMA-IR = homeostasis model assessment of insulin resistance; T-CHO = total cholesterol; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; TG = triglyceride; TP = total protein; Alb = albumin; AMY = amylase; BUN = blood urea nitrogen; Cre = creatinine; UA = uric acid; CRP = C-reactive protein.

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus the control group.

Although peripheral total leukocyte, neutrophil, lymphocyte, and monocyte counts did not correlate with FBG and HbA1c (fig. 2A, B) levels, they significantly correlated with insulin levels and HOMA-IR (fig. 2C, D).

Peripheral total leukocyte, neutrophil, lymphocyte. and monocyte counts did not correlate with T-CHO levels (fig. 3A); however, lymphocyte and monocyte counts positively correlated with LDL-C levels and negatively correlated with HDL-C levels (fig. 3B, C). Peripheral lymphocyte count significantly correlated with TG levels, but total leukocyte, neutrophil, and monocyte counts did not (fig. 3D).

Peripheral monocyte count, but not lymphocyte count, significantly correlated with visceral and subcutaneous fat thickness as measured by ultrasonography (fig. 4A, B) and with body fat mass and percentage body fat as calculated by a bioimpedance analysis device (fig. 4C, D).