Long or irregular menstrual cycles and risk of prevalent and incident non-alcoholic fatty liver disease

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Short title: Long or irregular menstrual cycles and NAFLD

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ABSTRACT

Context

The association of menstrual cycle length and irregularity with the risk of non-alcoholic fatty liver disease (NAFLD) is unknown. We examined this association in large cross-sectional and cohort studies.

Methods

The cross-sectional study included 72,092 women aged <40 years who underwent routine health examinations; the longitudinal analysis included the subset of 51,118 women without NAFLD at baseline. Long or irregular cycles were defined as menstrual cycles of ≥40 days or too irregular to estimate. Abdominal ultrasonography was performed to identify NAFLD. Multivariable Cox proportional hazard regression analyses were performed to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) for incident NAFLD according to menstrual cycle regularity and length, with 26–30-day cycles as the reference.

Results

At baseline, 27.7% had long or irregular menstrual cycles and 7.1% had prevalent NAFLD. Long or irregular menstrual cycles were positively associated with prevalent NAFLD. During a median follow-up of 4.4 years, incident NAFLD occurred in 8.9% of women. After adjustment for age, body mass index, insulin resistance and other confounders, the multivariable-adjusted HR for NAFLD comparing long or irregular menstrual cycles to the reference group was 1.22 (95% CI, 1.14–1.31); this association strengthened in the time-dependent analysis with HR of 1.49 (95% CI, 1.38–1.60).

Conclusions

Long or irregular menstrual cycles were associated with increased risk of both prevalent and incident NAFLD in young, premenopausal women. Women with long or irregular menstrual

cycles may benefit from lifestyle modification advice to reduce the risk of NAFLD and associated cardiometabolic diseases.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), the most common chronic liver disease worldwide (1), can progress to liver cirrhosis and hepatocellular carcinoma and is associated with higher risks of premature mortality (1). NAFLD is also strongly associated with insulin resistance (2), type 2 diabetes mellitus (2), and increased cardiovascular risk (1). Lifestyle modification continues to be the standard of care for NAFLD (3); hence, patients at risk may benefit from the assessment of easily identifiable risk factors for early intervention before progression to adverse outcomes. Reproductive factors and sex hormones have been suggested as risk factors for NAFLD because NAFLD is more prevalent among men than women and among postmenopausal women than premenopausal women (4).

Long or irregular menstrual cycles, which may be associated with metabolic or endocrine disorders (5-7), are common among women during the reproductive period, with a reported prevalence of approximately 20% (8). Long or irregular menstrual cycles are associated with cardiometabolic dysfunction, including insulin resistance (9), risk of type 2 diabetes mellitus (5), cardiovascular disease (6,10). However, the association between long or irregular menstrual cycles and NAFLD, a metabolic liver disease, has not been described previously. Evidence for an association between polycystic ovary syndrome (PCOS) and increased risk of NAFLD was inconsistent in a large meta-analysis (11). Long or irregular menstrual cycles are common in women with PCOS, which may occur as the ovarian manifestation of metabolic syndrome (12). However, PCOS is a heterogeneous condition that can be difficult to diagnose (13), and not all women with long or irregular menstrual cycles have PCOS. It is also unclear whether the strength of the association with NAFLD varies with the features of PCOS. Women with PCOS with hyperandrogenism had a higher risk of NAFLD than those with PCOS with normal androgen levels or those without PCOS (14,15);

however, no previous study has investigated whether menstrual cycle irregularity or oligomenorrhea are risk factors for NAFLD in women with PCOS. Additionally, no study has yet investigated the association between menstrual irregularity and NAFLD in the general population.

Hence, we aimed to examine the association between long or irregular menstrual cycles and NAFLD in a cohort of premenopausal women undergoing routine health examinations using cross-sectional and longitudinal study designs.

MATERIALS AND METHODS

Study population

The cohort of this study was derived from the Kangbuk Samsung Health Study and consists of premenopausal Korean women aged <40 years who underwent comprehensive annual or biennial health examinations at one of the healthcare centers in Seoul and Suwon, South Korea (16). The study subjects participated in comprehensive health examinations from 2011 to 2017, with at least one follow-up examination conducted by December 31, 2019 (N=135,090). We used data collected from routine health screening examinations, consisting of questionnaires, blood tests, imaging, and procedures (16). The exclusion criteria were as follows: history of liver disease or use of medications for liver disease (n=2,539); confirmed hepatitis B or C (n=3,177); liver cirrhosis (n=4); alcohol ≥20 g/day(17) (n=10,434); use of steatogenic medication within the previous year including amiodarone, tamoxifen, methotrexate, or corticosteroids (n=619); history of cancer (n=2,540); thyroid function abnormalities or use of medication for hyperthyroidism or hypothyroidism (n=4,262); premature menopause (n=233); previous oophorectomy or hysterectomy (n=2,592); use of hormone replacement therapy or contraceptives (n=4,153); pregnant or lactating (n=9,448);

and missing data on menstrual cycle, abdominal ultrasonography, alcohol intake, body mass index (BMI), and assessment of insulin resistance (n=44,547). Some participants met more than one exclusion criterion, resulting in 72,092 eligible women (**Figure 1**). For the longitudinal analysis of this cohort, we included subjects who were NAFLD-free at baseline with at least one follow-up visit; subjects with NAFLD at baseline (n=5,225) and who did not participate in follow-up examinations (n=17,165) were thus excluded. In total, 51,118 women were finally included in the study of incident NAFLD. This study was approved by the Institutional Review Board of Kangbuk Samsung Hospital (IRB No. KBSMC 2021-09-003), which waived the requirement for informed consent because we used a de-identified dataset retrieved from routine health screening examinations.

Data collection

Data on demographics, medical history, and behavioral factors were collected using standardized, self-administered questionnaires. Smoking status was categorized into never, former, or current smokers. Alcohol intake was assessed by estimating the amount of alcohol intake per day based on the frequency and amount consumed per drinking day. Physical activity was based on the Korean version of the International Physical Activity Questionnaire Short Form (18). Health-enhancing physical activity (HEPA) was defined as either vigorous activity ≥3 days per week accumulating ≥1,500 metabolic equivalent (MET)-min/week, or 7 days of walking or moderate to vigorous intensity activities accumulating ≥3,000 MET min/week (19). Education level was categorized as having a high school degree or lower or a college degree or higher. Parity was assessed based on the number of previous pregnancies, including live births and stillbirths. Early menarche was defined as menarche at <12 years (20). The women were asked whether their menstrual cycles were regular or too irregular to

estimate and were informed that cycles differing by 2–3 days could be considered regular; those with regular menstrual cycles were asked to report the interval in days. The menstrual cycles were categorized as follows: <21-day, 21–25-day, 26–30-day, 31–39-day, and ≥40-day or too irregular to estimate, similar to those in previous studies including the Nurses' Health Study II (5,21). Menstrual cycle lengths vary widely across populations of women, and while normal cycles can range from 21 to 35 days (22,23), the median menstrual cycle length in our cohort was 28 days; hence, the 26–30-day cycle (28 ± 2 days) was set as the reference group, as in other studies (24,25). However, considering that normal menstrual cycles are defined as 21 to 35 days (23), and that the Tremin Trust studies defined >40-day cycles as abnormally long menstrual cycles (26), we performed additional analysis by re-classifying the last two categories as 31–35-day, 36–40-day, and >40-day cycles or too irregular to estimate.

Participants' height, weight, and blood pressure (BP) were measured by trained nurses. Obesity was defined as BMI ≥25 kg/m^2, the cutoff value specified for diagnosing obesity in Asians (27). Hypertension was defined as systolic BP ≥140 mmHg, diastolic BP ≥90 mmHg, or reported use of any antihypertensive medication.

Blood samples were drawn from the antecubital vein after ≥10 hours of fasting for measurements of fasting blood glucose, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, alanine aminotransferase, aspartate transaminase, gamma-glutamyl transferase, and high-sensitivity C-reactive protein. Fasting insulin (Roche catalog No. 12017547, RRID:AB_2756877; http://antibodyregistry.org/AB_2756877) (28) was measured by electrochemiluminescence immunoassay using the Modular Analytics E170 during 2011–2014 and afterwards with the Cobas E602 Analyzer (Roche Diagnostics, Tokyo, Japan). Insulin resistance was determined based on the following homeostatic model assessment of insulin resistance (HOMA-IR)

188 equation: fasting blood insulin (μU/mL) × fasting blood glucose (mmol/l) / 22.5; and the
189 cutoff value of 2.5 was used (29). Diabetes mellitus was defined as fasting blood glucose
190 ≥126 mg/dL, hemoglobin A1c ≥6.5%, or reported use of any antidiabetic medication.
191 The diagnosis of NAFLD was based on hepatic steatosis identified on abdominal
192 ultrasonography performed by experienced radiologists blinded to the study aims. The
193 diagnosis of hepatic steatosis was based on standard criteria, including diffusely increased
194 fine echogenicity in the liver parenchyma compared to the kidney or spleen parenchyma,
195 deep beam attenuation, and bright vessel walls (30). Radiologists graded hepatic steatosis as
196 mild, moderate, or severe (31). Mild hepatic steatosis was identified by a slight increase in
197 liver echogenicity. Moderate hepatic steatosis was identified by a slightly impaired image of
198 the intrahepatic vasculature and diaphragm, accompanied by increased liver echogenicity.
199 Severe hepatic steatosis was identified by a marked increase in liver echogenicity, impaired
200 penetration of the posterior segment of the right lobe, and poor or no image of the
201 intrahepatic vasculature and diaphragm (32,33). NAFLD severity was categorized as mild or
202 moderate/severe in further analysis. The inter- and intra-observer reliability values for
203 diagnosing hepatic steatosis were substantial (kappa statistic 0.74) and excellent (kappa
204 statistic 0.94), respectively (34). The other requirements for diagnosing NAFLD were met
205 through the exclusion criteria applied at the beginning of this study, including the exclusion
206 of participants with significant alcohol intake, competing etiologies for hepatic steatosis, and
207 other causes of chronic liver diseases (3).
208 In a subsample of women who underwent pelvic ultrasonography examinations,
209 experienced gynecologists who were blinded to the study aims routinely questioned the
210 examinees regarding the diagnosis of gynecologic disorders, including PCOS, and examined
211 for the presence of ovarian cysts, including specific information on the size, echogenicity,

echotexture, internal pattern, and content. Previous gynecological disorders or abnormal findings on pelvic ultrasonography were documented in the ultrasonography reports.

Statistical analysis

Baseline characteristics were described according to menstrual cycle categories using descriptive statistics with adjustment for age, because age differs across the menstrual cycle categories.

Menstrual cycle and prevalent NAFLD: A cross-sectional study

We analyzed the association between menstrual cycle category and NAFLD by performing logistic regression analyses to calculate the prevalence ratios (PRs) and 95% confidence intervals (CIs) for NAFLD, with adjustment for age. We used two models for adjustment of covariates: Model 1 was adjusted for age, center (Seoul or Suwon), year of examination, alcohol consumption, smoking, physical activity, education level, parity, age at menarche, and BMI; Model 2 was adjusted for the variables in Model 1 plus HOMA-IR quintiles because insulin resistance is associated with both NAFLD (2) and PCOS (35). We adjusted for potential confounders that might affect the relationship between menstrual cycles and NAFLD. The confounding variables were defined using the following criteria: 1) causal association with the outcome (NAFLD); 2) non-causal or causal association with exposure (the menstrual cycle); and 3) not being a mediator in the causal pathway between exposure (the menstrual cycle) and the outcome (NAFLD). For the secondary analyses, we estimated the PRs and 95% CIs for mild and moderate/severe NAFLD for the menstrual cycle categories with no NAFLD as the reference group, using multinomial logistic regression models.

Menstrual cycle and incident NAFLD: A cohort study

The primary outcome was the development of NAFLD during follow-up among premenopausal women without NAFLD at baseline. The participants were followed up from baseline until the development of NAFLD or the end of 2019, whichever came first. Hazard ratios (HRs) and 95% CIs for the development of NAFLD were calculated using Cox proportional hazards regression analyses, with adjustment for variables as in logistic regression analyses. We also performed time-dependent analyses according to menstrual cycle category, smoking, alcohol consumption, physical activity, parity, BMI, and HOMA-IR quintiles as time-varying covariates. We tested for linear trends by applying menstrual category groups as continuous variables in the regression models. We also tested for a quadratic trend to allow for a J shaped relationship between menstrual cycle length and NAFLD by squaring the linear trend variable, which was centered on the reference value.

Predefined subgroup analyses were performed according to age (<30 vs. ≥30 years, since menstrual cycle length differs and decreases with age (36)), current smoking status, alcohol intake (<10 vs. ≥10 g/day, 10 g of ethanol per day for women as the cutoff of light drinking (37)), HEPA (no vs. yes), early menarche, parity, obesity defined using Asian specific criteria (BMI <25 kg/m^2 vs. ≥25 kg/m^2 (38,39)), HOMA-IR (<2.5 vs. ≥2.5 (29)), and high-sensitivity C-reactive protein (<1.0 vs. ≥1.0 mg/L, the proposed cutoff as an inflammatory marker for low risk of cardiovascular disease by the Center for Disease Control and American Heart Association (40)). We tested for interactions among the subgroups by performing likelihood ratio tests and compared the models with and without multiplicative interaction terms. We also performed analysis for prevalent and incident NAFLD among women who underwent pelvic ultrasonography examinations and excluded those with a

report of PCOS diagnosis or polycystic ovaries on ultrasonography findings.

Statistical analyses were performed using Stata version 16.0 (StataCorp LP, College Station, TX, USA). Two-tailed *p* values <0.05 were considered to indicate statistical significance.

Patient and public involvement

Patients and the public were not involved in the design, conduct, reporting, or dissemination of this research.

RESULTS

Characteristics of study participants

At baseline, 7.1% had prevalent NAFLD, while 27.7% had long (≥40-day) or irregular menstrual cycles. Compared with 26–30-day menstrual cycles as the reference, long or irregular menstrual cycles were associated with younger age, hypertension, diabetes, obesity, and higher total cholesterol, triglyceride, hsCRP, and HOMA-IR levels (**Table 1**).

Association between menstrual cycle and prevalent NAFLD

Table 2 shows the adjusted PRs of NAFLD based on menstrual cycle categories. Compared with 26–30-day menstrual cycles as the reference, <21-day, 31–39-day, and ≥40-day or irregular menstrual cycles were associated with a higher prevalence of NAFLD, with age-adjusted PRs (95% CIs) of 1.21 (1.07–1.36), 1.35 (1.29–1.41), and 1.89 (1.83–1.95), respectively. After additional adjustments for center, examination year, alcohol consumption, smoking status, HEPA, education level, parity, age at menarche, BMI, and HOMA-IR, 31–39-day and ≥40-day or irregular cycles were associated with NAFLD, with PRs (95% CIs) of

1.27 (1.19–1.36) and 1.35 (1.28–1.42), respectively. After categorizing NAFLD as mild or moderate/severe, 31–39-day and ≥40-day or irregular menstrual cycles were associated with a higher prevalence of mild and moderate/severe NAFLD in a dose-response manner compared to that of the reference group (**Supplemental Table 5**) (41). After reconfiguring the menstrual cycle categories as <21-, 21–25-, 26–30-, 31–35-, 36–40-, >40-day or irregular cycles, 31–35-day, 36–40-day, and >40-day or irregular cycles were also associated with NAFLD (**Supplemental Table 7**) (41).

Association between menstrual cycle and incident NAFLD

In the cohort analysis of women without NAFLD at baseline (**Supplemental Table 1**) (41), 4,524 incident cases of NAFLD occurred during a mean follow-up of 4.4 years. When analyzing the longitudinal associations between menstrual cycle categories and incident NAFLD, <21-day, 31–39-day, and ≥40-day or irregular menstrual cycles were associated with the development of NAFLD, with age-adjusted HRs (95% CIs) of 1.54 (1.19–1.99), 1.13 (1.03–1.24), and 1.26 (1.18–1.35), respectively (**Table 3**). The associations remained significant after adjusting for additional covariates, including HOMA-IR values. In the time-dependent analysis, 31–39-day and ≥40-day or irregular cycles were associated with a higher risk of incident NAFLD, with HRs (95% CIs) of 1.27 (1.15–1.39) and 1.49 (1.38–1.60), respectively, compared to the reference group. In a sensitivity analysis using moderate/severe NAFLD as an endpoint, ≥40-day or irregular menstrual cycles were associated with a higher risk of incident moderate/severe NAFLD in the fully-adjusted model and time-dependent analysis (**Supplemental Table 6**) (41). After reconfiguring the menstrual cycle categories, 21-day, 31–35-day, and >40-day or irregular cycles were associated with NAFLD risk in the fully adjusted model; in the time-dependent analysis, 31–35-day, 36–40-

day, and >40-day or irregular cycles were associated with increased NAFLD risk (**Supplemental Table 8**) (41).

In subgroup analyses, 31–39-day and ≥40-day or irregular menstrual cycles were associated with an increased risk of NAFLD when HOMA-IR was <2.5, with adjusted HRs (95% CIs) of 1.18 (1.08–1.30) and 1.27 (1.18–1.37), respectively, but not when HOMA-IR was ≥2.5 (*p* for interaction=0.001) (**Supplemental Table 2**) (41). No other significant interactions were observed for the other predefined subgroups.

Association between menstrual cycle and NAFLD among women with pelvic ultrasonography data

Among the women with pelvic ultrasonography data and gynecologic assessments available, after excluding 300 women with suspected PCOS, 18,968 women were included in the analysis at baseline. In the cross-sectional analysis, 31–39-day and ≥40-day or irregular menstrual cycles were associated with NAFLD, with adjusted PRs (95% CIs) of 1.28 (1.11–1.46) and 1.42 (1.27–1.58), respectively (**Supplemental Table 3**) (41).

In the longitudinal analysis of 14,378 women without either NAFLD or suspected PCOS at baseline, the adjusted HRs (95% CIs) for 31–39-day and ≥40-day or irregular menstrual cycles were 1.23 (1.03–1.47) and 1.32 (1.15–1.52), respectively (**Supplemental Table 4**) (41).

DISCUSSION

In our large cohort of premenopausal women, long or irregular menstrual cycles were associated with an increased risk of NAFLD compared to 26–30-day cycles, in both cross-sectional and longitudinal analyses. Long or irregular menstrual cycles were associated with a

higher prevalence of mild and moderate/severe NAFLD in a dose-response manner, and the association between long or irregular menstrual cycles and NAFLD risk was more pronounced in time-dependent analyses and were not fully explained by obesity, insulin resistance, or other relevant measured confounders in the subgroup analyses. Importantly, our results indicate that menstrual irregularity, which is easier to diagnose and usually presents earlier than PCOS (42), highlights the possibility of identifying premenopausal women at risk of developing NAFLD.

While limited evidence exists regarding how menstrual irregularities may affect the pathogenesis of NAFLD, PCOS has been linked with NAFLD in most of the previous studies included in the meta-analysis by Shengir et al (11). However, although women with long or irregular menstrual cycles are reported to be more likely to have hirsutism and ovulatory infertility than those with regular 26–31-day cycles (5), not all women with long or irregular menstrual cycles have PCOS. The prevalence of PCOS is reported to range from 4% to 21%, depending on the diagnostic criteria applied (43). In our study, 28% of participants had long or irregular menstrual cycles. Meanwhile, previous reports have suggested that the prevalence of PCOS in women with oligomenorrhea is estimated to be approximately 45%–87% (44,45), indicating that PCOS is present in a subgroup of women with abnormal menstrual cycles. On the other hand, women with PCOS and long menstrual cycles, or oligomenorrhea, have poorer metabolic profiles than those without oligomenorrhea (7,46,47). There is also a report that oligomenorrhea predicts the risk of diabetes mellitus in the absence of hyperandrogenism (5), potentially suggesting that menstrual cycle length may be the major determinant of metabolic abnormalities, beyond simply a proxy marker of PCOS. We lacked data to identify hyperandrogenism or hirsutism in our cohort; however, after excluding women with PCOS in our subgroup analysis of women with pelvic ultrasonography data and assessment by a

gynecologist, the association between long or irregular menstrual cycles and NAFLD persisted in both the cross-sectional and longitudinal analyses, suggesting that PCOS may not fully explain the relationship.

The association between long or irregular menstrual cycles and NAFLD remained after further adjustment for HOMA-IR in both cross-sectional and longitudinal analyses, as well as in time-dependent analysis. Long or irregular menstrual cycles were associated with an increased risk of NAFLD in the subgroup with less insulin resistance (HOMA-IR <2.5) but not in the subgroup with more insulin resistance (HOMA-IR ≥2.5), although there were only few women in the HOMA-IR ≥2.5 group. This suggests that insulin resistance, which has been posited to contribute to the association between PCOS and NAFLD,(11) does not fully explain the association between long or irregular menstrual cycles and NAFLD demonstrated in our study. Menstrual irregularity may also be a consequence of unhealthy lifestyle factors such as disordered eating and stress (48), which may increase the risk of NAFLD; however, there was no evidence for effect modification by factors such as smoking, alcohol consumption, HEPA, and obesity.

The prevalence of NAFLD in our study (7.0%) is similar to that reported previously for premenopausal women and represents a lower prevalence of NAFLD than that reported in general or male populations, which ranges from 20% to 42% (4). We attempted to exclude women experiencing the perimenopausal transition by excluding women aged >40 years, because hormonal changes that predispose women to NAFLD (49) could confound our analysis of menstrual cycles among premenopausal women. The higher risk of incident NAFLD compared to women with 26-30 day menstrual cycles observed among women with <21-day menstrual cycles in the longitudinal analysis may be attributable to perimenopausal changes, such as lower estradiol and high follicle-stimulating hormone levels(50) and shorter

regular menstrual cycles (22). However, this association was not statistically significant in the time-dependent analysis. Interestingly, <21-day menstrual cycles were also associated with higher age-adjusted PRs for NAFLD, higher proportions of diabetes and obesity, and higher mean HOMA-IR values compared to the reference group, as well as higher proportions of current smokers and participants with alcohol intake of 10–19 g/day. Polymenorrhea (<21-day menstrual cycles) may appear to be associated with poor metabolic profiles, similar to those with long or irregular menstrual cycles. However, <21-day menstrual cycles were not associated with NAFLD after adjusting for other covariates. Similarly, previous studies have shown that while oligomenorrhea and amenorrhea were associated with insulin resistance in women with PCOS, the association between polymenorrhea and insulin resistance was comparable to that of women with normal menstrual cycles (47,51).

While the mechanisms underlying the association between long or irregular menstrual cycles and NAFLD are unclear, exposure to estrogen may contribute to this association. Low 17β-estradiol levels, as well as the use of antiestrogens such as tamoxifen and aromatase inhibitors, have been associated with NAFLD (52). In contrast, estrogen replacement therapy has been reported to decrease the risk of NAFLD, which is reportedly twice as common in postmenopausal women than in premenopausal women (52,53). Estrogen is suggested to suppress inflammation; improve mitochondrial function; modulate nuclear receptors; and mitigate oxidative stress, insulin resistance, and fibrogenesis, to decelerate the progression of chronic liver diseases including NAFLD (54). The estrogen receptor alpha (ERα) expressed in the liver is suggested to contribute to hepatic sexual dimorphism (55) and lower the incidence of hepatic diseases in premenopausal women (56,57). In female mice, ERα was found to counteract the accumulation of lipids in the liver following excessive dietary fat intake, by inhibiting lipid synthesis and promoting

mitochondrial fatty acid β-oxidation (57). Although early menopause was not associated with NAFLD in a previous study (58), it may have lacked power due to the limited number of participants with early menopause. Further studies including larger populations are warranted to clarify the role of estrogen insufficiency and sex hormone abnormalities in the development of NAFLD.

Besides estrogen exposure, androgen excess and hypogonadotropic hypogonadism may contribute to the association between long or irregular menstrual cycles and NAFLD. Increased luteinizing hormone (59) and androgen (9) levels have been reported in women with irregular menstrual cycles. Normal androgen levels help balance fat and lean mass; conversely, hyperandrogenism may predispose to fat accumulation (53), especially in the abdomen (4). Moreover, hypogonadotropic hypogonadism is common in women who experience significant weight loss, exercise excessively, or are under severe stress and may manifest as long or irregular menstrual cycles (60). Although there was no significant interaction between menstrual length and obesity, the association between long or irregular menstrual cycles and NAFLD was more pronounced in non-obese women, while the association was not significant in obese women. Iron overload may also contribute to the association between long or irregular menstrual cycles and NAFLD; as increased hepatic iron was associated with NAFLD and progression of non-alcoholic steatohepatitis in some studies, regular menstruation may contribute to decreased NAFLD risk (61,62).

The strength of our study was the use of both cross-sectional and longitudinal study designs. Our study also has some limitations. First, the menstrual cycle was assessed using self-administered questionnaires. We attempted to avoid misclassification bias by including women aged <40 years and excluding older women who were more likely to be menopausal or who reported the use of estrogen replacement therapy or oral contraceptives. Second, we

did not have information on the participants' sex hormone or prolactin levels. Further studies with information on androgen and estrogen levels may help elucidate their influence on the association between menstrual cycles and NAFLD. However, the prevalence of hyperprolactinemia is reported as less than 1% of the general population (63,64); therefore, the overall findings may be less likely to be affected. Third, we could not identify the women meeting the criteria for a diagnosis of PCOS among all women with long or irregular menstrual cycles, because we did not have information on biochemical hyperandrogenism. However, our main findings remained consistent after excluding women with suspected PCOS using data from pelvic ultrasonography examinations and gynecologic assessments, which were available for one-fourth of the women in our cohort. Moreover, our aim was to assess long or irregular menstrual cycles as a risk factor for NAFLD in premenopausal women, regardless of a possible diagnosis of PCOS. Fourth, the diagnosis of NAFLD was based on ultrasonography, instead of histological diagnosis; the latter is the gold standard but is not appropriate for routine health screening examinations. Instead, ultrasonography is employed in epidemiological studies and provides reliable identification of NAFLD (65). Finally, because we included relatively healthy, young premenopausal Korean women, our results may not be generalizable to other populations with comorbidities, older age groups, or women of different ethnicities.

CONCLUSION

Our results indicate that long or irregular menstrual cycles may provide an easily identifiable marker for an increased risk of NAFLD in young, premenopausal women. Screening for NAFLD and counseling to promote healthy lifestyle behaviors may benefit women with a history of long or irregular menstrual cycles.

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Data availability: The datasets generated and analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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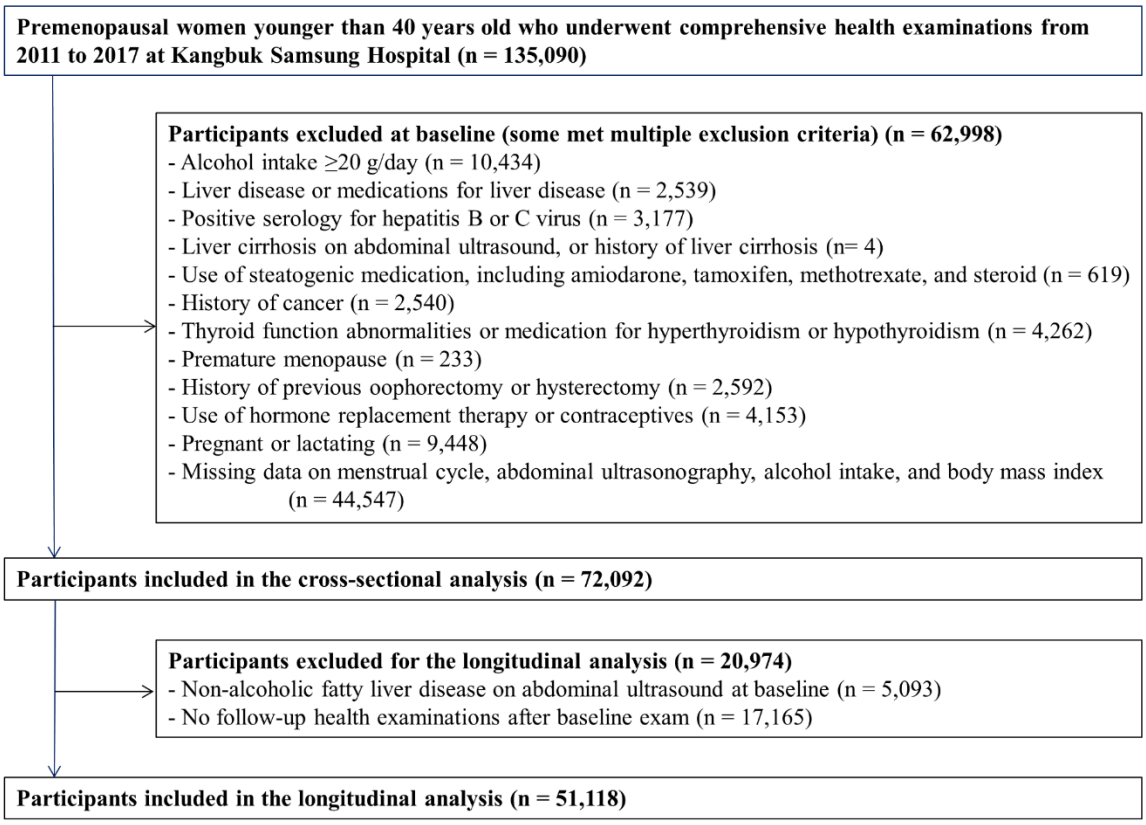
661 **FIGURE LEGEND**

662 **Figure 1** Selection of study participants



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665 **Table 1. Age-adjusted mean values (95% CI) and proportions (95% CI) of baseline characteristics by menstrual cycle category**

666 **(n=72,092)**

Characteristics	Menstrual cycle (days)					*p* for trend
	<21	21–25	26–30	31–39	≥40 or irregular	
Number	914	4,367	36,378	10,455	19,978	
Age (years)	31.3 (31.0-31.6)	33.5 (33.4-33.7)	33.0 (33.0-33.1)	32.0 (31.9-32.1)	31.9 (31.8-32.0)	<0.001
Seoul center (%)	51.6 (48.4-54.8)	58.4 (56.9-59.8)	57.9 (57.4-58.4)	58.5 (57.5-59.4)	55.5 (54.8-56.2)	<0.001
Current smoker (%)	4.5 (3.2-5.9)	2.2 (1.7-2.6)	2.0 (1.9-2.2)	1.8 (1.6-2.1)	2.6 (2.4-2.8)	0.074
Alcohol intake (%)[a]	14.8 (12.5-17)	12.9 (11.9-13.9)	12.5 (12.2-12.9)	12.5 (11.9-13.1)	13.8 (13.3-14.3)	0.001
HEPA (%)	14.5 (12.2-16.8)	12.1 (11.1-13.0)	10.9 (10.5-11.2)	10.4 (9.8-10.9)	12.0 (11.6-12.5)	0.133
High education level (%)[b]	60.5 (57.5-63.6)	77.9 (76.7-79.2)	83.6 (83.3-84.0)	86.6 (86.0-87.2)	79.1 (78.6-79.7)	0.085
Hypertension (%)	1.2 (0.4-1.9)	1.3 (1.0-1.7)	1.2 (1.1-1.3)	1.2 (1.0-1.4)	1.7 (1.6-1.9)	<0.001
Diabetes (%)	0.6 (0.1-1.1)	0.4 (0.3-0.6)	0.4 (0.4-0.5)	0.3 (0.2-0.5)	1.0 (0.9-1.2)	<0.001
History of CVD (%)	0.7 (0.1-1.2)	0.2 (0.1-0.3)	0.3 (0.2-0.3)	0.2 (0.1-0.3)	0.3 (0.2-0.4)	0.734
Lipid lowering drug (%)	0.1 (-0.1-0.4)	0.2 (0.1-0.3)	0.1 (0.1-0.1)	0.1 (0.1-0.2)	0.3 (0.2-0.4)	<0.001

Early menarche (%)	5.2 (3.8-6.6)	8.7 (7.8-9.5)	9.3 (9.0-9.6)	8.3 (7.8-8.8)	7.1 (6.7-7.4)	<0.001
Parous (%)	56.4 (53.5-59.3)	55.3 (54.0-56.5)	56.6 (56.2-57.1)	55.9 (55.1-56.7)	56.5 (55.9-57.0)	0.873
Obesity (%)[c]	13.1 (10.9-15.3)	8.0 (7.2-8.8)	8.5 (8.2-8.8)	8.8 (8.3-9.3)	12.2 (11.8-12.7)	<0.001
Body mass index (kg/m^2)	21.7 (21.5-21.9)	21.0 (20.9-21.1)	21.1 (21.0-21.1)	21.1 (21.0-21.1)	21.4 (21.4-21.5)	<0.001
Glucose (mg/dl)	90.0 (89.4-90.6)	89.6 (89.3-89.8)	89.6 (89.5-89.6)	89.6 (89.5-89.8)	90.5 (90.3-90.6)	<0.001
Total cholesterol (mg/dl)	180.4 (178.5-182.3)	178.9 (178.0-179.8)	179.9 (179.6-180.2)	181.9 (181.4-182.5)	184.3 (183.8-184.7)	<0.001
LDL-C (mg/dl)	105.1 (103.4-106.8)	102.5 (101.7-103.2)	103.8 (103.6-104.1)	106.2 (105.7-106.7)	107.6 (107.2-107.9)	<0.001
HDL-C (mg/dl)	66.9 (65.9-67.8)	68.2 (67.7-68.6)	67.5 (67.3-67.6)	67.1 (66.8-67.3)	66.6 (66.4-66.8)	<0.001
Triglycerides (mg/dl)	74.1 (71.6-76.6)	70.2 (69.1-71.4)	71.9 (71.5-72.3)	74.7 (74.0-75.5)	78.7 (78.2-79.3)	<0.001
AST (U/l)	17.2 (16.6-17.9)	17.7 (17.4-18.0)	17.6 (17.5-17.7)	17.9 (17.7-18.1)	18.5 (18.4-18.6)	<0.001
ALT (U/l)	13.8 (13-14.6)	13.9 (13.5-14.2)	13.7 (13.6-13.9)	14.1 (13.9-14.4)	15.5 (15.4-15.7)	<0.001
GGT (U/l)	14.7 (14.0-15.5)	13.9 (13.5-14.3)	14.3 (14.2-14.4)	14.7 (14.4-14.9)	16.1 (15.9-16.3)	<0.001
hs-CRP (mg/l)	0.78 (0.60-0.96)	0.75 (0.67-0.83)	0.77 (0.74-0.80)	0.79 (0.74-0.85)	0.94 (0.90-0.98)	<0.001
HOMA-IR	1.41 (1.33-1.49)	1.28 (1.24-1.31)	1.30 (1.28-1.31)	1.30 (1.27-1.32)	1.40 (1.38-1.42)	0.042

667 Abbreviations: ALT, alanine aminotransferase; AST, aspartate transaminase; CI, confidence interval; CVD, cardiovascular disease; GGT,

668 gamma-glutamyl transferase; HEPA, health-enhancing physical activity; HDL-C, high-density lipoprotein cholesterol; hs-CRP, high-

669 sensitivity C-reactive protein; HOMA-IR, homeostasis model assessment of insulin resistance; LDL-C, low-density lipoprotein cholesterol.

670 [a] ≥10 g of ethanol per day.

671 [b] ≥College graduate.

672 [c] Body mass index ≥ 25 kg/m^2.

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Table 2. Adjusted prevalence ratios (PRs) of non-alcoholic fatty liver disease (NAFLD) by menstrual cycle category at baseline (N= 72,092)

Menstrual cycle Length (days)	Number	Prevalent cases	Prevalence rate (%)	Age-adjusted PR[a] (95% CI)	Multivariable-adjusted PR[a] (95% CI)	
					Model 1	Model 2
<21	914	65	7.1	1.21 (1.07-1.36)	0.95 (0.75-1.14)	0.94 (0.75-1.13)
21-25	4,367	220	5.0	0.77 (0.72-0.81)	0.89 (0.80-0.99)	0.90 (0.80-0.99)
26-30	36,378	2,118	5.8	1.00 (reference)	1.00 (reference)	1.00 (reference)
31-39	10,455	749	7.2	1.35 (1.29-1.41)	1.30 (1.21-1.38)	1.27 (1.19-1.36)
≥40 or irregular	19,978	1,941	9.7	1.89 (1.83-1.95)	1.38 (1.31-1.45)	1.35 (1.28-1.42)
p for linear trend				<0.001	<0.001	<0.001
p for quadratic trend				<0.001	<0.001	<0.001

[a] Estimated from the logistic regression models. Multivariable Model 1 was adjusted for age, center, year of examination, alcohol consumption, smoking, physical activity, education level, parity, age at menarche, and BMI; Model 2: Model 1 plus adjustment for HOMA-IR quintile.

Abbreviations: BMI, body mass index; CI, confidence interval; HOMA-IR, homeostatic model assessment of insulin resistance.

Table 3. Development of non-alcoholic fatty liver disease (NAFLD) by menstrual cycle category at baseline (N= 51,118)

Menstrual period (days)	Person-years (PY)	Incident cases	Incidence density (/10^3 PY)	Age-adjusted HR (95% CI)	Multivariable-adjusted HR[a] (95% CI)		HR (95% CI)[b] in a model with time-dependent variables
					Model 1	Model 2	
<21	2,273	61	26.8	1.54 (1.19-1.99)	1.38 (1.06-1.78)	1.34 (1.04-1.73)	1.20 (0.94-1.54)
21–25	13,794	248	18.0	0.96 (0.84-1.09)	0.97 (0.85-1.10)	0.97 (0.85-1.1)	0.88 (0.78-1.00)
26–30	114,869	2,118	18.4	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
31–39	30,749	620	20.2	1.13 (1.03-1.24)	1.18 (1.08-1.30)	1.20 (1.09-1.31)	1.27 (1.15-1.39)
≥40 or irregular	64,503	1,477	22.9	1.26 (1.18-1.35)	1.22 (1.14-1.30)	1.22 (1.14-1.31)	1.49 (1.38-1.60)
p for linear trend				<0.001	<0.001	<0.001	<0.001
p for quadratic trend				<0.001	<0.001	<0.001	<0.001

[a] Estimated from Cox proportional hazards models. Multivariable Model 1 was adjusted for age, center, year of examination, alcohol consumption, smoking, physical activity, education level, parity, age at menarche, and BMI; Model 2: Model 1 plus adjustment for HOMA-IR quintile.

[b] Estimated from Cox proportional hazard models with menstrual cycle category, smoking, alcohol consumption, physical activity, parity, HOMA-IR, and BMI as time-dependent variables and baseline age, center, year of examination, education level, and age at menarche as time-fixed variables.

Abbreviations: BMI, body mass index; CI, confidence interval; HOMA-IR, homeostatic model assessment of insulin resistance; HR, hazard ratio.