

BMJ Open Associations between occupational relative aerobic workload and resting blood pressure among different age groups: a cross-sectional analysis in the DPhacto study

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ABSTRACT

Objective High levels of occupational physical activity (OPA) increase heart rate, blood pressure (BP) and the risk of hypertension. Older workers may be more vulnerable to high levels of OPA due to age-related degeneration of the cardiovascular system and cardiorespiratory fitness. This study investigates the association of relative aerobic workload (RAW) with resting BP and examines if this relation is moderated by age.

Design Cross-sectional epidemiological study.

Setting Data were collected among employees of 15 Danish companies in the cleaning, manufacturing and transport sectors.

Participants 2107 employees were invited for participation, of these 1087 accepted and 562 (42% female and 4% non-Westerns) were included in the analysis based on the criteria of being non-pregnant, no allergy to bandages, sufficient amount of heart rate data corresponding to ≥ 4 work hours per workday or 75% of average work hours, and no missing outcome and confounder values.

Primary and secondary outcome measures The primary outcome measure was BP.

Results Heart rate reserve was estimated from ambulatory 24-hour heart rate measures covering 2.5 workdays per participant (SD 1.0 day). Age significantly moderated the association between RAW and BP. Mean intensity and duration of high RAW ($\geq 30\%$ heart rate reserve) showed positive associations with diastolic BP and negative associations with pulse pressure (PP) among participants ≥ 47 years old. Tendencies towards negative associations between RAW and BP were seen among participants < 47 years old.

Conclusions Mean intensity and duration of RAW increased diastolic BP among participants ≥ 47 years old. Negative associations with PP may be due to healthy worker selection bias. Prevention of hypertension should consider reductions in RAW for ageing workers.

INTRODUCTION

High levels of occupational physical activity (OPA) increase the risk of cardiovascular disease (CVD) and mortality.^{1–4} High OPA

Strengths and limitations of this study

- The study continuously measured ambulatory heart rate for 2–4 days, which was used to calculate the relative aerobic workload by the minimum heart rate and estimated maximal heart rate.
- Individual aerobic capacity was included in the assessment of physical workload.
- Resting blood pressure data were collected at one time point, and therefore we could not explore the acute domain-specific effects on blood pressure during and after work.
- Ambulatory blood pressure measurements, which have greater predictive validity for cardiovascular disease outcomes, were not available.
- Generalisability of the results is limited to the occupational groups included.

raises heart rate (HR), by the muscular work needed for performance, and an elevated HR is an independent risk factor for CVD.⁵ Elevated HR during OPA may therefore be one mechanism explaining why high OPA increases the risk for CVD.⁶ HR varies by age, level of cardiorespiratory fitness, health status, body temperature, working posture, and type, duration and intensity of OPA. Resting HR increases with age, while maximum HR and aerobic capacity decrease with age.^{7,8} Therefore, identical physical work task demands strain older workers to a higher degree than younger workers.⁹

The intensity of OPA can be assessed by the relative aerobic workload (RAW), estimated as percent heart rate reserve (%HRR), calculated from resting HR, working HR and age-predicted maximum HR.¹⁰ RAW has been used to investigate the association between OPA and CVD,^{11–13} and high RAW has been shown to increase the risk for

atherosclerosis,³ incidence of CVD,¹¹ all-cause and cardiovascular mortality,¹¹ and blood pressure (BP) elevation.¹⁴ Based on previous experimental studies, it is shown that BP is positively related to RAW through cardiac output, meaning that OPA raises HR, which increases cardiac output and thereby BP.¹⁵ However, the authors are only aware of one field study among cleaners investigating the relation between BP and RAW,¹⁴ and to verify these results the present study was designed.

BP elevations may be another mechanism explaining the association between high OPA and CVD due to the linear dose–response association between BP and CVD.¹⁶ Also, some studies indicate occupational lifting to increase BP^{17 18} due to the repeated acute rises in BP when performing the lifting.¹⁹ Prolonged exposure to elevated BP confers excessive mechanical strain on the arterial wall, possibly causing endothelial injury, smooth muscle cell proliferation and inflammatory reactions leading to atherosclerosis and CVD.^{20 21} With increasing age and increases in cumulative mechanical stresses, the arteries gradually lose compliance and thereby the ability to dilate sufficiently, increasing peripheral resistance and leading to higher BP, augmented by the effects of an early return of pulse wave reflection.^{15 22}

However, to our knowledge, only one previous study has investigated the association between occupational RAW and resting BP using objective HR field measures.¹⁴

The objectives of this study were to investigate whether occupational RAW was associated with resting BP, and secondly to investigate if this relation is moderated by age. The hypothesis is that RAW is positively associated with resting BP and that this association is stronger among older workers.

METHODS

Study design and population

Data from the Danish PHysical ACTivity cohort with objective measurements (DPhacto)²³ were used for this study. This study uses baseline data on OPA and BP from DPhacto. Fifteen companies in the cleaning, transport and manufacturing sectors were enrolled from December 2011 to March 2013 in collaboration with a large Danish labour union.²³ The companies were included if they allowed measurements during paid working hours. A total of 2107 recruited participants provided their written informed consent prior to participation. Baseline measures included questionnaires, measures of anthropometrics, BP, cardiorespiratory fitness and diurnal measurements of HR. Pregnant women were excluded from all measurements, and participants with allergy to bandages were excluded from the objective diurnal measurements.²³ DPhacto was conducted according to the Helsinki Declaration²⁴ and approved by the Danish Data Protection Agency and local ethics committee (H-2-2012-011).

Patient and public involvement

The participants were not actively involved in the study design, procedures or analyses. The union representing

the enrolled workers participated in the recruitment of some of the enrolled companies.²⁵

Assessment of exposure

RAW was assessed by the relative HR equation²⁶ as %HRR¹²—a proxy for maximal oxygen consumption (VO_2max).^{10 13} HRR was based on estimated age-adjusted maximal HR¹⁰ and HR during work, objectively measured with an HR monitor (Actiheart, CamNtech, Cambridge, UK).^{10 26} Actiheart measures the raw electrocardiographic signals with a sensitivity of 0.25 mV and calculates the HR from the R peaks in the QRS complex of the ECG. The participants were asked to wear the Actiheart 24 hours a day for 4 continuous days. The Actiheart is field-validated²⁷ and frequently used for HR field monitoring.¹² The Actiheart was mounted directly on the skin by pregelled electrodes (Ambu BlueSensor VL-00-S/25, Ambu, Ballerup, Denmark) at one of the validated positions.²⁸

HR measurements were limited to workdays, and measures were included in the analysis if they covered at least four continuous hours/day or $\geq 75\%$ of the average wear time during work across days per participant, including breaks, and had a beat error of 50% or less.²⁸

Participants were asked to fill in a diary stating the time at work and in bed, and the remaining time periods were analysed as leisure time and therefore includes time spent on chores and transportation activities. Accordingly, 24 hours were divided into periods of work, leisure and bedtime. The mean %HRR at work for all measured days was calculated as the mean %HRR per day measured at the participant level, and the mean across all measured days of %HRR during work was then weighted by the measured hours of work on separate days. In addition, the duration of work exceeding the recommended maximum 30% RAW during an 8-hour workday was extracted from the HRR.^{13 29}

Assessment of outcome

Resting BP was measured at a worksite health check, during paid working hours, performed by trained personnel. BP was measured three times, after sitting at rest for 10 min, using Omron M6 Comfort (Omron Healthcare, Kyoto, Japan), while the participant was asked to keep quiet and sit still. The average of the three measurements for systolic BP (SBP) and diastolic BP (DBP) was used in the analysis. In addition pulse pressure (PP) was calculated as the average of individual differences between SBP and DBP.^{15 30} SBP values < 80 and > 240 mm Hg and DBP values < 50 and > 130 mm Hg were considered physiological outliers and were excluded from analyses.¹⁴

Assessment of covariates

Age and sex were determined using a questionnaire. Country of birth was determined by the question 'In which country were you born?' and dichotomised into Western country (all European countries, Australia, Canada and USA) or non-Western country. Smoking was assessed by the question 'Do you smoke?' and dichotomised into yes

('yes daily', 'yes sometimes') or no ('used to smoke', 'I have never smoked'). Body mass index (BMI, kg/m²) was calculated from body weight (in kilogram; Tanita BC418) and height (in metre; Seca model 123 1721009). Cardiorespiratory fitness was estimated by the one-point Astrand bike ergometer test.³¹ Use of prescribed medication was determined by the questions 'Have you in the last three months been taking prescribed medication?' and 'If yes, what kind of medication? – Anti-hypertensive - Heart medication or Anti-depressives'. Occupational group was determined by the workplace of the participant and whether the participant stated to be working in administration (white-collar work) or in production (blue-collar work), thereby representing four groups: cleaning, manufacturing, transportation and administration (merged by administrative staff across occupational groups). OPA was determined by the question 'How would you describe your physical activity in your working hours?' with four response categories ('Mostly sedentary-no physical demands', 'Mostly standing or walking-otherwise no physical demands', 'Standing and walking with lifting or carrying', and 'Strenuous physical work'). Rate of perceived exertion at work³² was determined by the question 'How physically demanding do you normally consider your present work?' with a 10-scaled response category (1–10), where 10 was the most demanding. The extent of shift work was determined by the question 'At which time of the day do you usually work in your main occupation?' with the following response categories: 'Fixed day work', 'Night/Varying working hours with night' or 'Other'. Work hours per week was assessed by the question 'How many hours per week do you work in your main occupation, incl. extra hours?'. Seniority was determined by the question 'For how long have you had the kind of occupation as you have now?'. Occupational pushing, pulling, lifting and carrying were assessed by the question 'How much of your working time do you push or pull/carry or lift?' with a 6-point response scale from 1 ('almost all the time') to 6 ('never'). Intensity of leisure time physical activity (LTPA) was assessed by %HRR. The mean %HRR was normalised to the total of the included measured leisure time.

Statistical analysis

All statistical analyses were performed using SAS V.9.4. Associations between %HRR and BP (mm Hg) were estimated by linear regression analysis. Participants with missing data were excluded. The aforementioned covariates were tested for multicollinearity by variance inflation. Due to occurrence of multicollinearity, the covariates were excluded from the adjusted model. Covariates were included in the adjusted regression models using the change-in-effect method with inclusion of those covariates that changed the observed effect size (beta coefficient) of OPA more than 5% when entered in the multivariate model. In addition, age moderation was tested by a multiplicative interaction term; if the interaction term was significant ($p < 0.10$), analyses stratified by median age were considered to be the adequate analysis. The estimates from the stratified analysis were evaluated

based on the magnitude of differences between strata and their clinical significance.

To test the robustness of the findings, sensitivity analyses were additionally adjusted for educational level (skilled or unskilled).

RESULTS

Study population

A total of 562 participants, 326 men and 236 women, aged 18–68 years constituted the final study sample (figure 1). The mean seniority in the current or similar occupation was 13.3 years (SD 10.3 years). On average, HR measurements were conducted for 2.5 workdays per participant (SD 1.0 day). Additional characteristics of the study population are shown in table 1.

Construction of statistical model

The first model estimated the unadjusted association between exposure and outcome. The multicollinearity diagnostics did not lead to any exclusion of covariates. The following covariates met the 5% effect-change criterion and were included in the adjusted regression analyses (model 2): sex, country of birth, smoking, work hours per week, alcohol consumption, BMI and LTPA. The added interaction term (aerobic workload (%HRR) × age) in the adjusted models was significant ($p < 0.001$) for all outcomes. Therefore analyses were stratified by median age (<47 years old/≥47 years old) and were considered as the primary analysis. Moreover, to reduce any possible residual confounding by age, we included age as a covariate in all stratified models.

Age-stratified analysis

Among participants <47 year old, mean RAW and work hours ≥30%HRR were negatively associated with SBP, DBP and PP; however, in the adjusted models, none of these relationships was statistically significant. Among participants ≥47 years old, a 10% increase in mean RAW was associated with a statistically significant 2.2 mm Hg increase in DBP and a 3.1 mm Hg decrease in PP; each hour of work at ≥30%HRR was associated with an increase of 0.7 mm Hg in DBP and a decrease of 0.5 mm Hg in PP.

Sensitivity analyses

Sensitivity analyses additionally adjusting for educational level showed similar patterns (results not shown). Sensitivity analyses, excluding those using prescribed medication for hypertension, heart diseases and depression (n=84), showed a tendency towards lower SBP, with the exception of DBP among participants ≥47 years old, but no substantial changes among those younger than 47 years.

DISCUSSION

The association between RAW and BP differs by age group and by specific BP outcome measure. Among participants

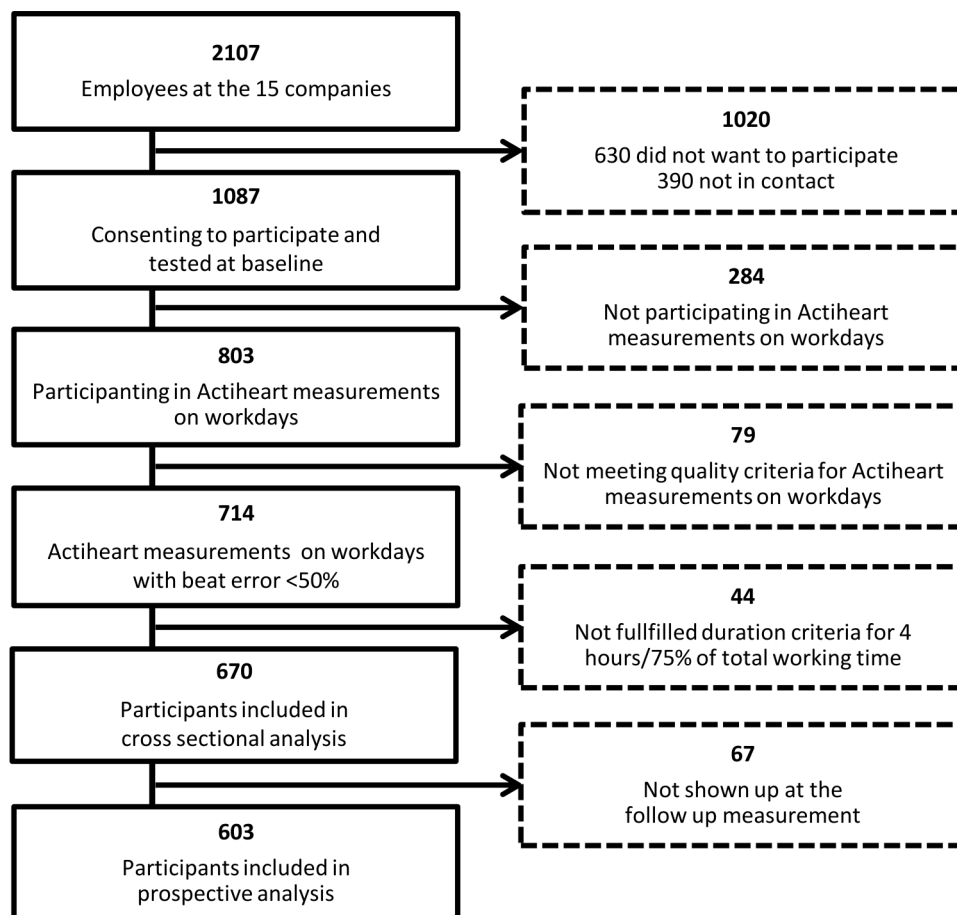


Figure 1 Flow of the participants.

aged ≥ 47 years, exposure to higher RAW increases DBP and decreases PP.

RAW may increase DBP,¹⁴ possibly because OPA is performed in an upright position (table 1). Upright positions increase venous pooling in the lower extremities, leading to decreases in central vascular plasma volume, which are compensated by increases in cardiac output, HR and especially DBP, to maintain sufficient BP for continuous perfusion of the upper body and brain.¹⁵

The observed pattern with substantial DBP increases and smaller decreases in SBP led to a decrease in PP. Physiologically, PP describes the relation between stroke volume and arterial compliance.¹⁵ Previously, aerobic exercise has been shown to increase arterial compliance and thus decrease PP³³; our PP results among older participants are consistent with these findings. However, the reduction in PP in our study was primarily due to an increase in DBP and much less due to a decrease in SBP. Therefore, and also because of the strong recent epidemiological evidence that higher levels of OPA—in contrast to LTPA—may be hazardous to cardiovascular health,³⁴ our results on PP reduction need to be interpreted with caution and do not indicate that higher levels of RAW will necessarily be cardioprotective.

Theoretically, older age impacts the relationship between RAW and BP by the age-dependent lowered aerobic capacity,¹⁰ leading to an increased RAW by

performance of the same work.^{7 8} Additionally, the age-dependent progression of arteriosclerosis and overall decreased arterial compliance both contribute to increases in the total peripheral resistance, leading to increases in BP.^{15 22} Thus, older participants are expected to be more vulnerable to increases in RAW and related increases in HR and BP. Our results show that participants aged ≥ 47 years have higher DBP, while at the same time their PP appears to be inversely related to RAW (table 2), which is in contrast to previous literature.^{15 22} Thus, the lower PP across the subgroup of participants aged ≥ 47 years needs to be interpreted with caution.

The observed effects across participants aged ≥ 47 years are more susceptible to healthy worker effect survivor bias,³⁵ originally describing actively employed to have lower mortality rates, than the population as a whole, due to the fact that unhealthy individuals more often leave the workforce.³⁶ Similarly, migrations out of high OPA jobs are expected for workers not physically capable to maintain employment due to deterioration of health. Consequently, the healthy worker survivor bias reflects a selection of healthier workers into high OPA jobs and thereby attenuating any estimated disease risks in this group. The ‘surviving’ healthy workers may have maintained better arterial compliance and could therefore experience less increases in the total peripheral resistance and BP than those that migrate out of high OPA jobs with

Table 1 Baseline characteristics of the study population, by age group

	Total population (N=562)			Population aged ≥47 years old (n=261)			Population aged <47 years old (n=301)		
	Mean	SD	n (%)	Mean	SD	n (%)	Mean	SD	n (%)
Age (years)	45.2	10.0		53.6	4.2		37.9	7.5	
Sex (female)			236 (42)			117 (45)			119 (40)
Country of birth (non-Western)			25 (4)			9 (3)			16 (5)
Current smoker			165 (29)			66 (25)			99 (33)
Body mass index (kg/m ²)	27.3	4.8		27.5	4.3		27.1	5.1	
Overweight (body mass index ≥25 kg/m ²)			366 (65)			186 (71)			180 (60)
Cardiorespiratory fitness (mLO ₂ /min/kg)	31.9	8.9		29.8	7.5		33.3	9.5	
Systolic blood pressure (mm Hg)	134.0	14.4		138.0	14.2		130.5	13.7	
Diastolic blood pressure (mm Hg)	83.9	10.0		86.2	9.3		81.9	10.2	
Pulse pressure (mm Hg)	50.1	11.0		51.9	11.4		48.6	10.4	
Hypertension (≥140/≥90 mm Hg and/or using antihypertensives)			231 (41)			129 (49)			102 (34)
Using one or more of prescription medication (antihypertensive, antidepressive or heart medication)			83 (15)			61 (23)			22 (7)
Antihypertensive			17 (3)			11 (6)			1 (1)
Antidepressive			16 (3)			6 (3)			6 (3)
Heart medication			67 (12)			44 (24)			10 (6)
Occupational group									
Cleaning			91 (16)			50 (19)			41 (14)
Manufacturing			384 (68)			169 (65)			215 (71)
Transport			53 (9)			25 (10)			28 (9)
Administration			34 (6)			17 (7)			17 (6)
Educational level (skilled)			183 (33)						
Subjectively rated intensity of occupational physical activity (scale 1–4)						76 (29)			107 (36)
1: mostly sedentary-no physical demands			91 (16)			32 (17)			27 (15)
2: mostly standing or walking-no physical demands			103 (18)			36 (19)			34 (19)
3: standing and walking with lifting or carrying			257 (46)			83 (45)			85 (47)
4: strenuous physical work			79 (14)			23 (12)			25 (14)
Lifting and carrying at work (scale 1–6)	3.7	1.4		3.7	1.4		3.6	1.5	
Lifting and carrying ≥50% of time			224 (40)			101 (39)			123 (41)
Pushing and pulling at work (scale 1–6)	3.8	1.4		3.9	1.3		3.7	1.4	
Pushing and pulling ≥50% of time			198 (35)			81 (31)			117 (18)
Rate of perceived exertion at work (scale 1–10)	5.8	2.2		5.8	2.2		5.7	2.2	
Seniority in current or similar occupation (years)	13.6	10.3		17.2	11.5		10.5	7.8	
Work hours per week	38.1	5.2		37.4	4.8		38.7	5.5	
Shift work (night shift)			78 (14)			33 (13)			45 (15)

Continued

Table 1 Continued

	Total population (N=562)			Population aged ≥47 years old (n=261)			Population aged <47 years old (n=301)		
	Mean	SD	n (%)	Mean	SD	n (%)	Mean	SD	n (%)
Intensity of physical activity during leisure (%HRR)	25.6	5.1		25.3	5.5		25.6	4.6	
Total duration of included measurements of working hours (hours)	18.7	7.7		19.3	7.7		18.3	7.5	
Duration of included working hours per day (hours/day)	7.6	1.3		7.6	1.3		7.6	1.3	
Relative aerobic workload (%HRR) during working hours	30.0	7.5		29.9	7.8		30.0	7.2	
Mean %HRR ≥30 during working hours			282 (50)			126 (48)			154 (51)
Working hours ≥30%HRR (hours/day)	3.0	1.8		3.0	1.9		3.0	1.7	

%HRR, per cent heart rate reserve.

less compliant arteries. A lower BP and a lower HR due to higher aerobic capacity among younger healthier workers may enable these selected workers to cope better with the high OPA than would be expected from older workers, or the general population including many retirees or persons with disabling conditions. This could also explain why the PP decreases among surviving workers aged ≥47 years old. Yet the decrease in PP may also be explained by the notion that increases in SBP are thought to lead to angina pectoris.³⁷ This would lead workers with coronary heart disease to leave high OPA jobs and thus generate a healthy worker survivor bias. The healthy worker survivor bias may therefore be especially pronounced among

populations including workers with diagnosed hypertension or heart disease. However, our sensitivity analysis excluding those participants using prescription medicine for hypertension, heart diseases and depression (n=84) showed results similar to the primary results.

To our knowledge, only one previous study investigated the association between RAW and BP.¹⁴ This study showed positive relations between RAW and BP. One explanation for these contradictory findings could be the difference in the timing of the BP measurement. In the current study, we studied resting BP, which is only measured at one time point, whereas the former study measured ambulatory BP every 20 min during waking time,¹⁴ which

Table 2 Adjusted associations of 10% increments in mean relative aerobic workload (%HRR) and hours of work at ≥30%HRR with SBP, DBP and pulse pressure, by age group (<47 years old, n=301; ≥47 years old, n=261)

Age group		SBP			DBP			Pulse pressure		
		mm Hg	95% CI	P value	mm Hg	95% CI	P value	mm Hg	95% CI	P value
10% changes in mean relative aerobic workload										
<47 years old	Model 1	-2.29	-4.44 to -0.14	0.04	-0.47	-2.07 to 1.14	0.57	-1.83	-3.47 to -0.19	0.03
	Model 2	-1.65	-3.77 to 0.46	0.12	-0.66	-2.26 to 0.93	0.41	-0.99	-2.75 to 0.77	0.27
≥47 years old	Model 1	-1.28	-3.51 to 0.95	0.26	2.19	0.74 to 3.64	<0.01*	-3.47	-5.22 to -1.72	<0.01
	Model 2	-0.84	-3.62 to 1.93	0.55	2.23	0.43 to 4.02	0.02*	-3.07	-5.18 to -0.95	<0.01
Duration of relative aerobic workload ≥30%HRR										
<47 years old	Model 1	-0.73	-1.62 to 0.16	0.11	-0.05	-0.71 to 0.61	0.88	-0.68	-1.36 to -0.01	0.05
	Model 2	-0.44	-1.32 to 0.43	0.32	-0.01	-0.66 to 0.65	0.99	-0.44	-1.16 to 0.29	0.24
≥47 years old	Model 1	-0.06	-0.98 to 0.86	0.90	0.84	0.24 to 1.43	<0.01*	-0.90	-1.63 to -0.17	0.02
	Model 2	0.14	-0.91 to 1.20	0.79	0.68	-0.01 to 1.36	0.05	-0.53	-1.35 to 0.28	0.20

Model 2 is adjusted for age, sex, country of birth, smoking, work hours per week, alcohol consumption, body mass index and HRR intensity during leisure time physical activity.

DBP, diastolic blood pressure; %HRR, per cent heart rate reserve; SBP, systolic blood pressure.

better captures the immediate short-term responses of RAW to BP during workday. Even if higher RAW does not increase resting BP, it may still increase BP during work and the average 24-hour BP, which would increase CVD risk despite small decreases in resting BP. Future prospective investigations of the effects of RAW on 24-hour ambulatory BP, cumulative HR and other cardiovascular risk factors are needed to better understand the mechanisms explaining previous findings showing that 10% increases in RAW increase all-cause mortality by 13%, coronary heart disease mortality by 28% and acute myocardial infarction by 18%.^{11 12}

Practical implications

Occupational groups exposed to high RAW suffer from increased risks of CVD.^{1-4 11} To develop targeted interventions for the prevention of hypertension and CVD among working populations, better understanding of the aetiological mechanisms is needed. Our results show that mean levels and duration of high RAW are positively associated with increases in DBP, especially among participants ≥ 47 years old. These results indicate that among the older subgroup, it would take less than a 10% rise in the mean RAW or approximately 176 min during work at an intensity of $\geq 30\%$ HRR to increase DBP by 2 mm Hg, a change that is being considered clinically significant at the population level.^{38 39} Hence, our results indicate potential for primary prevention of hypertension and CVD by keeping OPA below the recommended maximum level of RAW, that is, below 30%HRR.^{13 29}

Methodological considerations

A strength of this study is the continuously measured HR for 24 hours/day for 2–4 days. The HR measures were used to calculate the minute-by-minute RAW, by the measured minimum HR and estimated maximal HR,¹⁰ thus including the individual aerobic capacity into the assessment of physical workload. RAW was calculated both as a mean across a workday and as the number of working hours spent in excess of the recommended maximum intensity of 30%HRR.

In the stratified analysis, the estimates were evaluated based on their differences in the subgroups as well as their clinical significance.

However, the study also has some limitations. No power calculation was performed initial to this study, which might explain the weak associations reported. We only had one-time resting BP data and therefore could not explore the acute domain-specific effects on BP during and after work. Moreover, ambulatory BP has been shown to be of greater predictive validity for CVD outcomes than resting BP.^{40 41} Also, the measured resting BP may overestimate the individual level of PP due to the usual greater rise in SBP than DBP during such measurements.⁴² Thus, future studies should strive to collect ambulatory BP. The generalisability of the results is limited to the occupational groups included.

CONCLUSION

These cross-sectional associations of mean daily RAW and work hours at $\geq 30\%$ HRR with BP showed that the effects of RAW on BP differ by age group and by specific BP outcome measure. Among older but not younger workers, exposure to higher RAW increases DBP measures and decreases PP.

These observations indicate more complex relationships between RAW and age as previously assumed and call for additional research examining various pathophysiological mechanisms that might explain the observed increased risks for hypertension and CVD among workers exposed to high levels of RAW.

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Contributors MBJ and AH conceived the idea for the cohort and supervised the collection of data and building of the database, as well as discussed and revised the protocol for this study and the manuscript critically. MK conceived the idea for this study, wrote the initial protocol for analysis, carried out the analysis and drafted the manuscript. EC, NK and NG interpreted and discussed the results and revised the protocol for analysis and manuscript critically. All authors are accountable for all aspects of the work and have approved the final version of the manuscript.

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