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Expedited CO₂ respiration in people with Miltenberger erythrocyte phenotype GP.Mur

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In Southeast Asia, Miltenberger antigen subtype III (Mi.III; GP.Mur) is considered one of the most important red blood cell antigens in the field of transfusion medicine. Mi.III functions to promote erythrocyte band 3 expression and band 3-related HCO₃⁻ transport, with implications in blood CO₂ metabolism. Could Mi.III affect physiologic CO₂ respiration in its carriers? Here, we conducted a human trial to study the impacts of Mi.III expression in respiration. We recruited 188 healthy, adult subjects for blood typing, band 3 measurements, and respiratory tests before and after exercise. The 3-minute step exercise test forced the demand for CO₂ dissipation to rise. We found that immediately following exercise, Mi.III+ subjects exhaled CO₂ at greater rates than Miltenberger-negative subjects. Respiration rates were also higher for Mi.III+ subjects immediately after exercise. Blood gas tests further revealed distinct blood CO₂ responses post-exercise between Mi.III and non-Mi.III. In contrast, from measurements of heart rates, blood O₂ saturation and lactate, Mi.III phenotype was found to be independent of one's aerobic and anaerobic capacities. Thus, Mi.III expression supported physiologic CO₂ respiration. Conceivably, Mi.III+ people may have advantages in performing physically enduring activities.

Miltenberger antigens are glycoprotein variants that belong to the complex MNS blood group system^{1,2}. There are 11 glycoprotein variants in the Miltenberger series (termed Mi.I to Mi.XI). From structural analyses, they presumably evolved from homologous gene recombination events between glycoprotein A (GPA) and glycoprotein B (GPB)³. Besides Mi.III, all the other Miltenberger antigens have extremely low occurrence frequencies worldwide, and are considered rare erythrocyte antigens in blood banking. The incidence of Mi.III is only 0.012% among Caucasians, and even lower among northern Chinese and Japanese^{2,4-6}; its occurrence rates range between 2–6% in most parts of Taiwan and other Southeast Asian (SEA) regions^{5,7}. GP.Mur is the protein entity of Mi.III phenotype. Because GP.Mur is highly antigenic, it is considered one of the most important red blood cell (RBC) antigens in blood banking in SEA^{7,8}.

In human erythrocytes, GPA and band 3 are two most abundant membrane proteins, each with approximately 10⁶ copies in an average erythrocyte. Band 3, also known as anion exchanger-1 (AE1), facilitates HCO₃⁻/Cl⁻ bidirectional transport across the red cell membrane. This anion exchange activity of band 3 plays an essential role in blood CO₂ respiration¹². Blood CO₂ exists mostly in the form of HCO₃⁻, and the conversion between CO₂ and HCO₃⁻ is mediated by carbonic anhydrase (CA) inside RBCs¹². Band 3 serves as a gate on the RBC membrane for HCO₃⁻ to enter or exit erythrocytes. Because the rate of HCO₃⁻ conductance by band 3 is at least one order of magnitude slower than the rate of CA-mediated conversion, the anion transport activity of band 3, rather than the enzyme activity of carbonic anhydrase, is the rate-limiting factor for blood CO₂ respiration¹².

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MALE	Number of healthy subjects ¹	Age (yrs)	Height (cm)	Weight (Kg)	BMI ²	Ideal Body Weight (Kg) ³	Exercise Frequencies (times/wk)	Blood Pressure (mmHg) ⁴	
								Systolic	Diastolic
<i>Non-Mi.III</i>	46	27.3 ± 9.1	172.6 ± 5.0	72.3 ± 11.0	24.3 ± 3.7	68.3 ± 4.5	2.0 ± 1.6	118.2 ± 13.7**	71.2 ± 11.0 [#]
<i>Mi.III</i>	24	31.1 ± 10.0	174.0 ± 3.8	75.5 ± 12.1	25.0 ± 4.0	69.6 ± 3.5	2.4 ± 1.6	130.0 ± 13.0**	81.2 ± 11.6 [#]
FEMALE									
<i>Non-Mi.III</i>	63	33.3 ± 10.6	158.5 ± 5.9	55.9 ± 7.0	22.3 ± 2.6	55.5 ± 5.3	1.7 ± 1.4	113.2 ± 11.4*	72.7 ± 9.9
<i>Mi.III</i>	46	35.3 ± 10.0	162.0 ± 4.7	60.2 ± 8.5	23.0 ± 3.4	58.7 ± 4.3	1.4 ± 1.3	121.5 ± 13.9*	73.7 ± 10.8

Table 1. Initial physical assessment for the healthy subjects participated in this study⁵. ¹Adult participants were deemed “healthy” subjects by the following criteria: BMI ≤ 30 (non-obese), and free from asthma, diabetes, hypertension, cardiovascular diseases, cancer, bone-and-joint problems, or major viral infection (HBV/HCV), and not in the Registry for Catastrophic Illness under the Taiwan National Health Insurance Program. ²BMI, body-mass index. ³Ideal body weight was calculated using the Devine equation³⁸. ⁴Blood pressures were measured in quietness, prior to the exercise test. Statistically significant differences were found among subjects with the Mi.III phenotype and those without. ** $p < 0.005$; [#] $p < 0.005$; * $p < 0.01$. ⁵Data were presented in mean ± S.D.

GPA serves as a chaperone that is responsible for the expression of band 3^{13–16}. GPB, a structural homologue of GPA, expresses one-fifth the quantity of GPA on the red cell surface, and is functionally unclear^{14,17}. In people bearing the Mi.III blood type, half or all of their GPB protein molecules on the RBC membrane are replaced by GP.Mur¹⁸. GP.Mur, encoded for Mi.III, is a naturally-occurred hybrid protein comprised of GPB and GPA. The sequence of the *GYP.Mur* gene contains a fragment of *GYP.A* inserted in the sequence of *GYP.B*¹⁹, and this unique glycoprotein B-A-B hybrid structure is capable of promoting band 3 protein production and surface expression, just like GPA¹⁸. Our previous *ex vivo* study identified superior band 3-related functions (e.g. larger $\text{HCO}_3^-/\text{Cl}^-$ transport capacities) in Mi.III + RBCs^{18,20}. Since people with the Mi.III blood type express more band 3 on their red cells, their capacity of CO_2 respiration were expected to be larger^{18,20}.

The highest occurrence frequency of Mi.III worldwide is among the Taiwan Ami tribespeople (88%)⁸. Anecdotally, in Taiwan, Ami tribespeople are generally recognized for their athleticism and superior physical endurance, compared to people of other ethnic origins²¹. A disproportionately high percentage of the Amis are professional or elite athletes^{22,23}. Ami people represent only 0.6–0.7% of the Taiwanese population, but over 50% of Taiwanese track athletes who had won international competitions were Amis²². Conceivably, these general impressions regarding the Ami people (athleticism and superior physical endurance) might be associated with the Miltenberger blood type that most of them bear.

The main goal of this study was to determine the impacts of Mi.III blood type in respiration through a large-scale human study. Faster CO_2 expiration after a brief, physical challenge was observed in people with the Mi.III blood type. Ami tribespeople lacking the Mi.III phenotype did not have this respiratory advantage, indicating that the involvement of other tribal genes or factors is unlikely.

Results

In this study, we recruited 266 non-athlete adults for questionnaire survey, physical assessment, blood tests (including Miltenberger phenotyping, band 3 measurements, and complete blood count), and a 3-minute step test (a standardized cardiorespiratory fitness test). The total sample number was down to 188, after excluding subjects who failed to complete the 3-minute exercise test, or who have one or more of the following conditions: hypertension, BMI > 30, cardiovascular disease(s), asthma, HBV/HCV hepatitis, joint/bone problems, cancer(s), or other major diseases. Among the selected, physically-healthy subjects, 39% are positive with the Mi.III blood type. Their initial physical assessment and RBC-relevant complete blood count data were summarized in Tables 1 & 2. Mi.III and the non-Mi.III participants were not distinguishable in most of the categories in these two Tables. On the other hand, Mi.III+subjects had slightly higher blood pressure than non-Miltenberger subjects in general (Table 1). Additionally, compared to non-Mi.III female, Mi.III+female subjects had in average smaller sizes of red cells (Table 2, MCV) and less hemoglobin per RBC (Table 2, MCH). The smaller MCH values in Mi.III+female were likely due to their smaller RBC sizes. The differences in MCV and MCH between Mi.III and non-Mi.III female however were not observed among the male subjects (Table 2). Because the differences in MCV and MCH were restricted to female, they were unlikely due to the expression of Mi.III. The gender differences in red cell sizes were probably related to other Ami-unique genetic factors, as many of our Mi.III+subjects are Ami tribespeople. These variations in blood pressure, MCV and MCH were still within the normal ranges

MALE (subject number)	RBC count (10 ⁶ /μL)	Hb (g/dL)	HCT (%)	MCV ¹ (fL)	MCH ² (pg/cell)	MCHC (g/dL)	RDW (%)	RDW-SD (fL)
<i>Non-Mi.III</i> (46)	5.1 ± 0.4	14.9 ± 0.9	45.4 ± 2.4	89.8 ± 6.7	29.5 ± 2.7	32.8 ± 0.8	13.5 ± 0.8	41.4 ± 3.2
<i>Mi.III</i> (24)	5.0 ± 0.3	14.7 ± 0.9	45.3 ± 3.4	90.6 ± 3.1	29.6 ± 1.3	32.7 ± 0.6	13.5 ± 0.6	41.6 ± 2.6
FEMALE								
<i>Non-Mi.III</i> (63)	4.4 ± 0.4	12.9 ± 1.0	38.9 ± 5.3	90.3 ± 7.4 [#]	29.5 ± 2.9*	32.6 ± 0.7	14.2 ± 1.9	44.0 ± 4.1
<i>Mi.III</i> (46)	4.5 ± 0.4	12.5 ± 1.4	38.6 ± 3.7	85.2 ± 10.6 [#]	27.8 ± 3.7*	32.3 ± 0.8	14.7 ± 2.8	42.6 ± 3.4

Table 2. A summary of the results of complete blood count (CBC) test for the subjects³. ¹MCV (mean corpuscular volume), the average volume of a RBC in fL, was significantly different among Mi.III+ and Mi.III-negative female subjects ([#] $p < 0.01$), but not among the male subjects. ²MCH (mean corpuscular hemoglobin), the average quantity of hemoglobin in a RBC, had significant variations between Mi.III and non-Mi.III in female (^{*} $p < 0.05$), but not in male. ³Data were presented in mean ± S.D.

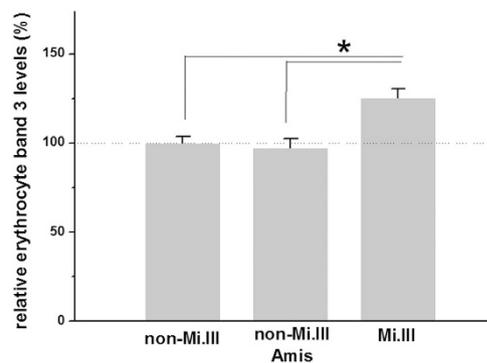


Figure 1. Mi.III+RBCs expressed more band 3 than Miltenberger-negative red cells. Relative band 3 expression was determined by DIDS labeling, and then normalized with respect to the average of non-Mi.III data (set 100%). Shown mean ± SEM. ^{*} $p < 0.001$.

Higher band 3 expression in Mi.III+subjects. Mi.III-encoded GP.Mur enhances the expression of erythrocyte band 3¹⁸. Here, we used a DIDS labeling method to verify band 3 expression levels in the red cells of these subjects. DIDS is a fluorescent compound that binds covalently and specifically to lysine-539 of band 3²⁴. By DIDS labeling, we found that Mi.III+RBC samples expressed in average 25.2% more band 3 than the blood type-negative samples (Fig. 1), which is in agreement with our previous measurements by quantitative iTRAQ mass spectrometry, Western blot, and flow cytometry^{18,25}. Because many of our Mi.III+subjects are Ami, Ami tribespeople who do not express Mi.III (“non-Mi.III Amis” in Fig. 1) were compared. As shown in Fig. 1, non-Mi.III Amis expressed significantly lower levels of band 3 than Mi.III+subjects. These results confirmed the direct association between Mi.III expression and higher band 3 levels, and excluded the possibility that other Ami-unique genes could also promote the expression of band 3.

Expedited CO₂ expiration following exercise in Mi.III+subjects. To determine the impacts of Mi.III in respiration, the subjects were asked to participate in a standardized 3-minute step test, and their cardiorespiratory parameters (end-tidal CO₂, breathing rates, heart rates, and blood O₂ saturation) were monitored before and immediately after the test. This 3-minute exercise test evaluates one’s cardiorespiratory fitness levels, and is backed up by a large population database on fitness collected by Taiwan National Council on Physical Fitness & Sports²⁸.

Among the physiological parameters measured, expired or end-tidal CO₂ (Figs. 2,3) and respiration rates (Fig. 4) immediately following the step test were distinguishable between Mi.III and non-Mi.III. Mi.III+test participants generally exhaled CO₂ faster right after the step test than Miltenberger-negative participants (Fig. 2). Because the amount of exhaled CO₂ (as expressed in end-tidal CO₂ or EtCO₂ in the figures) varied greatly from individual to individual (data not shown), the minute-to-minute changes of CO₂ expiration (as expressed in ΔEtCO₂/min) were calculated per individual, and then grouped for comparison (Fig. 2B). From the 30th second to the 1st minute post-exercise, there was 4.0 ± 0.8 mmHg drop of exhaled CO₂ from Mi.III+male participants and 2.6 ± 0.8 mmHg drop of EtCO₂ from non-Mi.III participants. From the 1st to the 2nd minute after exercise, the changes of CO₂ expiration became 2.8 ± 0.8 mmHg for Mi.III+male participants, and 5.0 ± 0.6 mmHg for non-Mi.III male participants

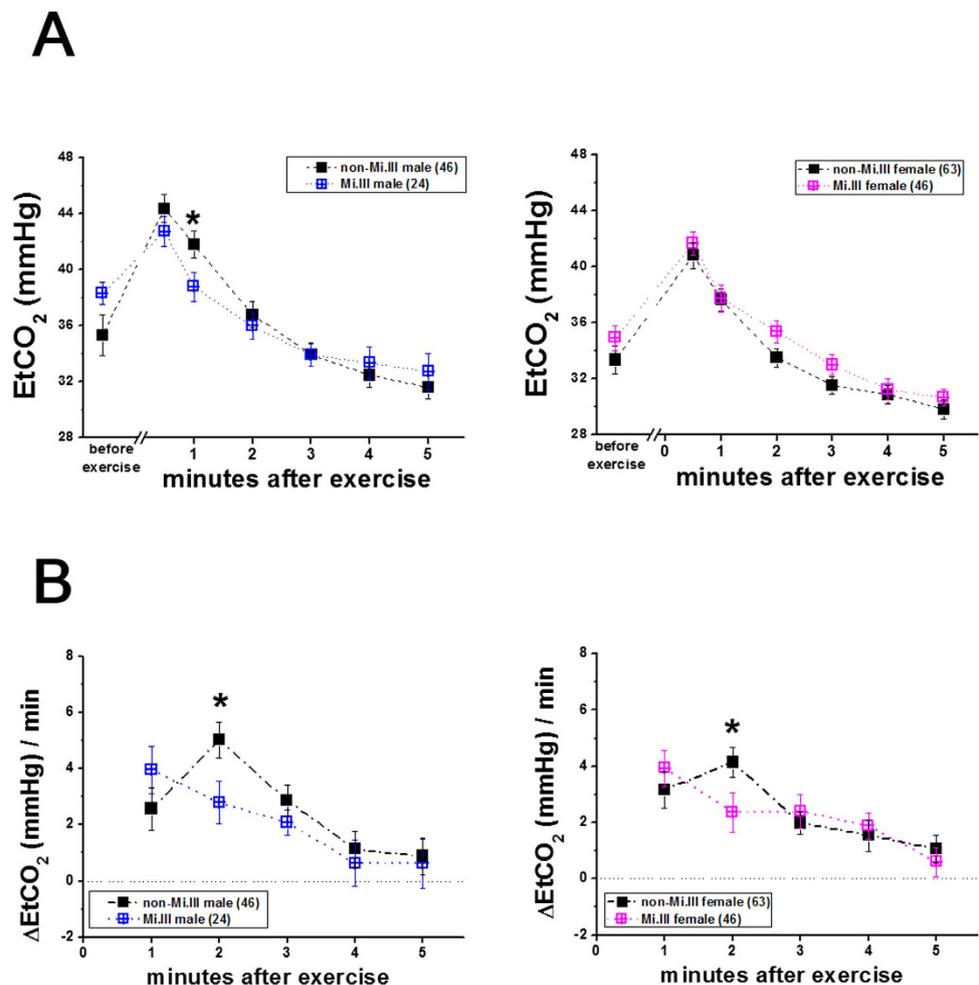


Figure 2. CO₂ clearance immediately following exercise were faster for Mi.III+subjects than for subjects lacking Mi.III expression. Left: EtCO₂ measurements in male participants; right: in female participants. (A) EtCO₂ measurements for Mi.III (blue or pink color) and non-Mi.III (black). (B) Minute-to-minute changes of CO₂ expiration (Δ EtCO₂/min) were compared between Mi.III (blue/pink) and non-Mi.III (black). CO₂ expiration in non-Mi.III participants remained substantial at the 2nd minute following the step test, but had subdued in Mi.III+participants by the 2nd minute (* $p < 0.05$). The numbers of the test subjects are indicated in parentheses. Shown mean \pm SEM.

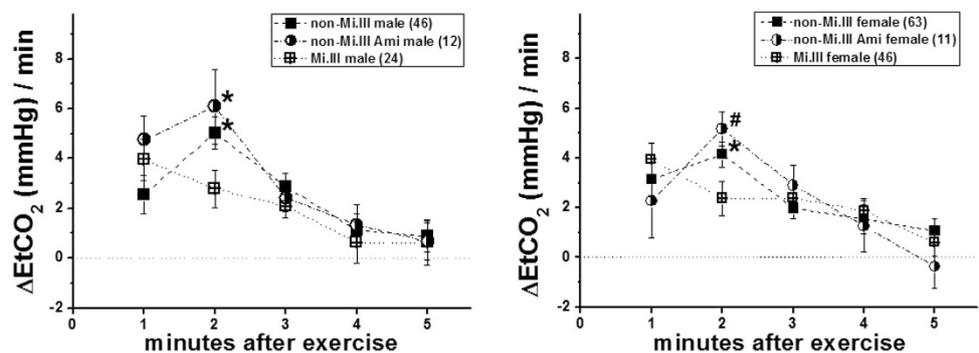


Figure 3. Expedited CO₂ expiration following the step test was unique to Mi.III, and not to Miltenberger-negative Ami people. The data for Mi.III+subjects are shown in cross symbols; for non-Mi.III subjects, in solid symbols; and for Miltenberger-negative Ami people, in half-solid symbols. Shown mean \pm SEM. * $p < 0.05$. # $p < 0.01$.

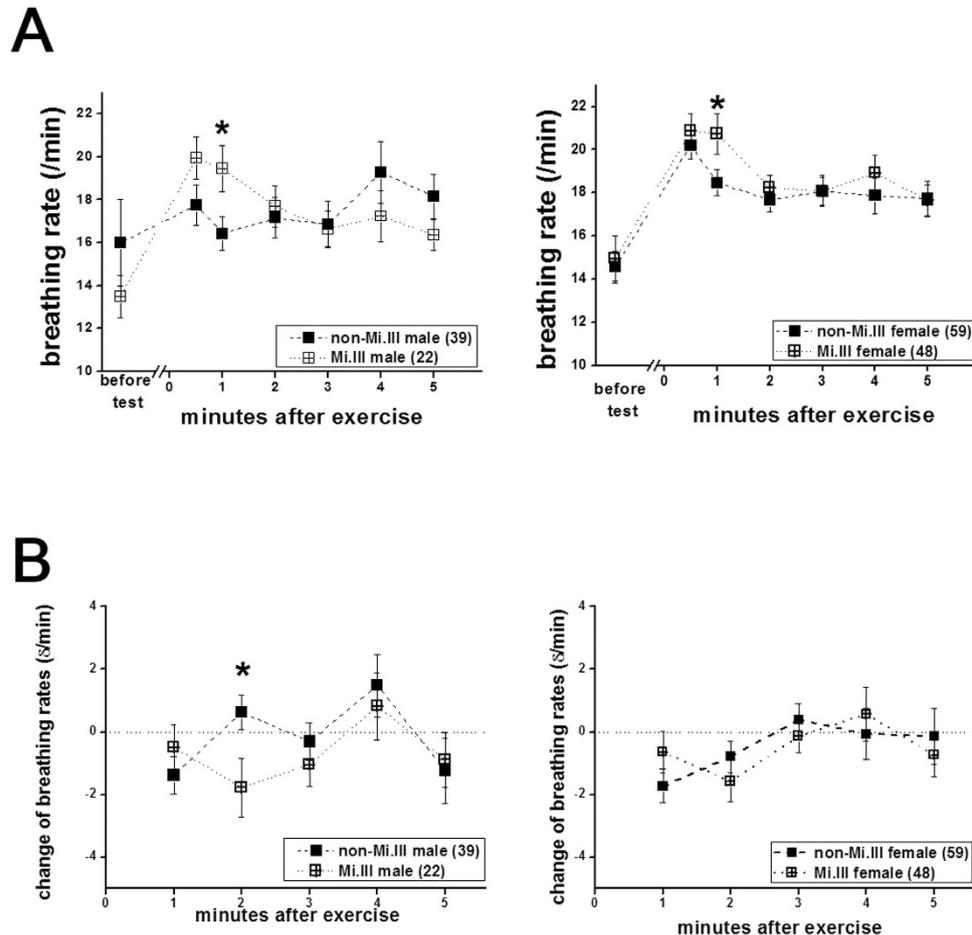


Figure 4. Breathing rates were also significantly higher in Mi.III+ subjects immediately following the step test. Left: data from the male participants; right: from the female participants. (A) Breathing rates for Mi.III (cross symbols) and non-Mi.III (solid symbols) immediately before and after the exercise test. Faster respiration was observed within the first minute after exercise in Mi.III than in non-Mi.III subjects, regardless of their gender. The differences in breathing rates subdued at later measured time points. (B) Minute-to-minute changes of breathing rates. Shown mean \pm SEM. * $p < 0.05$.

(Fig. 2B, left). That is, the rate changes of CO_2 expiration for Mi.III+ male had decreased by the 2nd minute post-exercise, but that for Miltenberger-negative male participants remained substantial (Fig. 2B). Similar kinetic differences in CO_2 expiration between Mi.III and non-Mi.III female were observed. From the 1st to the 2nd minute post-exercise, $\Delta\text{EtCO}_2/\text{min}$ for Mi.III+ female subjects had already dropped to 2.4 ± 0.7 mmHg, compared to 4.1 ± 0.5 mmHg at the same time interval for non-Mi.III female (Fig. 2B, right). For both male and female, the kinetic differences in CO_2 expiration ($\Delta\text{EtCO}_2/\text{min}$) between Mi.III and non-Mi.III diminished by the 3rd minute following exercise.

By comparing the differences in CO_2 expiration kinetics between male and female, we also found slightly faster recovery in male than in female after the exercise challenge. At the 4th minute post-exercise, $\Delta\text{EtCO}_2/\text{min}$ have dropped to 0.6 ± 0.8 mmHg for Mi.III male and 1.1 ± 0.7 mmHg for non-Mi.III male, indicating plateauing CO_2 expiration kinetics (Fig. 2B, left). When $\Delta\text{EtCO}_2/\text{min}$ approaches zero, there would be no more change in the rate of CO_2 expiration. On the other hand, for female subjects, $\Delta\text{EtCO}_2/\text{min}$ were 1.9 ± 0.5 mmHg (Mi.III) and 1.5 ± 0.6 mmHg (non-Mi.III) at the 4th minute post-exercise, and continued to drop (Fig. 2B, right).

To exclude other Ami-related factors than the Mi.III phenotype, we analyzed the data obtained from Ami subjects who lack Miltenberger expression. It is estimated that ~12% of Ami people do not express Mi.III blood type⁸. We found that the non-Mi.III Ami subjects showed significantly slower CO_2 clearance than Mi.III+ subjects, regardless of their gender (Fig. 3). Thus, expedited CO_2 expiration following exercise was unique to people with Mi.III expression, and appeared unrelated to other Ami factors.

Besides CO_2 expiration, breathing rates immediately following exercise were also significantly higher for Mi.III+ subjects, regardless of their gender (Fig. 4). The differences in breathing rates between Mi.III and non-Mi.III diminished by the 2nd minute following the test (Fig. 4A). We also calculated the minute-to-minute changes of breathing rates, and found that the changes between Mi.III and non-Mi.

	Quiet HR (/min)	Cardio-respiratory Endurance Index (%)	Predicted Max HR ¹ (/min)	HR (% max HR) at 30 th sec post-test	HR (% max HR) at 1 st min post-test	HR (% max HR) at 2 nd min post-test	HR (% max HR) at 3 rd min post-test	HR (% max HR) at 4 th min post-test	HR (% max HR) at 5 th min post-test
MALE									
<i>Non-Mi.III</i> (46)	74.7 ± 14.7	65.9 ± 18.8	228.0 ± 6.2	119.0 ± 17.8	104.8 ± 18.5	94.5 ± 18.7	91.0 ± 17.7	91.8 ± 16.5	90.7 ± 16.1
				(52.4 ± 7.9)	(46.2 ± 8.2)	(41.7 ± 8.2)	(40.2 ± 7.7)	(40.2 ± 7.2)	(40.0 ± 7.1)
<i>Mi.III</i> (24)	80.5 ± 12.9	60.9 ± 10.1	231.2 ± 7.5	121.2 ± 14.8	108.8 ± 15.1	99.9 ± 17.9	94.3 ± 17.6	97.1 ± 17.4	94.9 ± 15.5
				(52.4 ± 6.5)	(47.3 ± 6.5)	(43.4 ± 8.1)	(41.0 ± 7.9)	(42.4 ± 7.7)	(41.4 ± 6.9)
FEMALE									
<i>Non-Mi.III</i> (63)	78.3 ± 10.7	57.5 ± 9.6	231.9 ± 7.1	135.0 ± 18.7	118.7 ± 18.7	105.2 ± 16.7	98.5 ± 14.9	95.0 ± 13.6	93.7 ± 13.9
				(58.3 ± 8.5)	(51.3 ± 8.3)	(45.1 ± 7.4)	(42.2 ± 6.5)	(40.7 ± 6.0)	(40.2 ± 6.1)
<i>Mi.III</i> (46)	80.0 ± 12.1	55.3 ± 8.2	232.6 ± 7.0	135.8 ± 16.1	121.4 ± 18.4	108.6 ± 15.9	102.8 ± 14.2	99.3 ± 14.3	97.4 ± 13.8
				(59.0 ± 7.4)	(52.7 ± 8.3)	(46.5 ± 7.4)	(44.1 ± 6.7)	(42.7 ± 6.6)	(41.9 ± 6.3)

Table 3. Heart rates (HR) before and immediately following the stepping exercise test². ¹Predicted maximal heart rates = 209.6 – (0.67 × age [in years])²⁹. ²Data were presented in mean ± S.D.

	%SpO ₂ in quietness	%SpO ₂ at 30 th sec post-test	%SpO ₂ at 1 st min post-test	%SpO ₂ at 2 nd min post-test	%SpO ₂ at 3 rd min post-test	%SpO ₂ at 4 th min post-test	%SpO ₂ at 5 th min post-test
MALE							
<i>Non-Mi.III</i> (46)	98.6 ± 0.7	97.7 ± 1.9	98.1 ± 1.3	98.6 ± 0.6	98.8 ± 0.5	98.7 ± 0.5	98.7 ± 0.5
<i>Mi.III</i> (24)	99.0 ± 0.0	97.7 ± 1.2	98.3 ± 0.8	98.7 ± 1.3	98.5 ± 1.3	98.7 ± 0.6	98.8 ± 0.6
FEMALE							
<i>Non-Mi.III</i> (63)	98.8 ± 0.5	97.7 ± 2.3	98.7 ± 0.6	98.8 ± 0.5	98.8 ± 0.4	98.8 ± 0.5	98.8 ± 0.4
<i>Mi.III</i> (46)	99.0 ± 0.2	98.3 ± 0.9	98.7 ± 0.7	98.9 ± 0.4	98.9 ± 0.3	98.9 ± 0.3	98.8 ± 0.4

Table 4. Blood O₂ saturation (%SpO₂) before and immediately following the exercise test¹. ¹Data were presented in mean ± S.D.

III male were significantly different during the interval from the 1st to the 2nd minute post-exercise (Fig. 4B, left). The kinetics of breathing rates for Mi.III+ male had already dropped significantly by the 2nd minute post-exercise (Fig. 4B, left). On the other hand, the female subjects, unlike the male, did not show significant differences in the kinetics of breathing rates between Mi.III and non-Mi.III (Fig. 4B, right); this is probably because the differences between Mi.III and non-Mi.III female were relatively small (Fig. 4A). Breathing frequencies are primarily controlled in response to changes of blood CO₂ concentrations by the respiratory center in the central nerve system³³. By comparing the changes of breathing rates and CO₂ expiration (Figs. 2,4), their time course correlated well. By the 2nd minute post-exercise, both changes in EtCO₂ and in breathing rates decreased significantly in Mi.III+, as compared to the non-Mi.III (Figs. 2,4).

Aerobic capacity not associated with Mi.III expression. The two other cardiorespiratory parameters measured before and immediately after exercise—heart rate and blood O₂ saturation (SpO₂), did not differ between Mi.III and non-Mi.III (Tables 3 & 4). Heart rate is an important indicator for one's aerobic fitness²⁷. One's cardiorespiratory endurance index (CREI) score for each test participant could be calculated using three heart rate measurements after the step test, and be compared with the CREI norm charts published online by the Section of Physical Fitness & Sports of the Taiwan Sports Administration²⁸. Mi.III and non-Mi.III participants exhibited similar levels of cardiorespiratory fitness (Table 3). Besides, the percent maximal heart rates (% max HR) at any time points measured post-exercise (from the 30th second to the 5th minute following the step test) also did not differ significantly between Mi.III and non-Mi.III (Table 3)²⁹. So there appeared no associations between Mi.III expression and one's cardiorespiratory fitness.

As for blood O₂ saturation, there was a slight drop to 97.7–98.3% within 30 seconds post-exercise for all the subjects (Table 4). Their SpO₂ levels recovered within 1 minute to the pre-test levels, and remained relatively the same from the 2nd to the 5th minute post-exercise in all 4 groups (Mi.III male/non-Mi.III male/Mi.III female/non-Mi.III female). There were also no significant variations in SpO₂ observed at any time points before and after exercise between Mi.III and non-Mi.III (Table 4). In sum, these results suggested that Mi.III expression only expedited CO₂ respiration (Fig. 2) and did not affect aerobic capacity (Tables 3 & 4).

		mean	SD	mean	SD	Significance ³
		Non-Mi.III male (20) ¹		Mi.III male (10)		
venous CO ₂ (mmHg)	before	55.9	7.8	57.5	7.0	<i>n.s.</i>
	after	53.2	7.5	57.8	5.9	<i>n.s.</i>
	Difference ²	-2.69	4.80	0.30	2.91	* <i>P</i> < 0.05
pH	before	7.36	0.04	7.35	0.03	<i>n.s.</i>
	after	7.34	0.03	7.33	0.04	<i>n.s.</i>
	difference	-0.02	0.03	-0.02	0.03	<i>n.s.</i>
HCO ₃ ⁻ act (mM)	before	30.9	2.7	31.8	2.5	<i>n.s.</i>
	after	28.4	3.6	29.7	2.2	<i>n.s.</i>
	difference	-2.42	2.02	-2.07	0.77	<i>n.s.</i>
base excess (mM)	before	4.61	1.77	4.76	2.11	<i>n.s.</i>
	after	2.02	2.97	2.40	2.07	<i>n.s.</i>
	difference	-2.59	2.15	-2.36	0.72	<i>n.s.</i>
lactate (mg/dL)	before	12.5	3.5	14.6	5.2	<i>n.s.</i>
	after	27.6	14.5	32.5	11.0	<i>n.s.</i>
	difference	15.2	14.9	18.0	7.6	<i>n.s.</i>

Table 5. Blood gas and lactate immediately before and after the stepping test. ¹The number inside parentheses indicated the number of blood test subjects. ²“Difference” referred to the change of the measured values due to stepping for individual subjects; the difference was one’s post-exercise value subtracted by his pre-exercise value. Shown here were mean±S.D. for each parameter. ³To test if the pre-exercise measurements alone (or the post-exercise measurements alone) were significantly different between non-Mi.III and Mi.III groups, *t*-test was used. Another statistical method—ANCOVA was used to assess differences between the pre-exercise and the post-exercise measurements for each subject, and to determine whether Mi.III significantly affected the parameter following exercise. “*n.s.*”, not significant.

Distinct blood CO₂ responses following exercise in Mi.III versus non-Mi.III. We further examined how Mi.III erythrocyte expression could affect one’s CO₂ respiration by performing additional blood tests immediately before and after 3-minute step exercise on 30 male subjects. The tests included venous blood gas tests (CO₂, pH, HCO₃⁻ and base excess) and lactate measurements, and the results were summarized in Table 5. For all the subjects tested, 3-minute exercise resulted in small changes in venous CO₂, HCO₃⁻, and pH. This was expected, as the standardized 3-minute stepping test is considered moderate for healthy adults. For most of our test subjects, their heart rates remained below 60% HR_{max} immediately after exercise (Table 3). Anaerobic respiration in untrained people generally begin at 50–60% HR_{max}³⁰, so it was not surprising that there were very small increments of plasma lactate found immediately after the step test (Table 5).

We used analyses of covariate (ANCOVA) to test if any of the 5 parameters listed in Table 5 (CO₂, pH, HCO₃⁻, base excess, and lactate) were significantly affected by Mi.III expression following the exercise challenge. We found exercise-induced changes of venous CO₂ to be significantly different between Mi.III and non-Mi.III subjects (**P* < 0.05 by ANCOVA in Table 5). The other blood gas parameters and lactate were not affected by Mi.III expression, according to ANCOVA. On the other hand, by comparing the pre-exercise measurements between Mi.III and non-Mi.III subjects, or comparing their post-exercise blood measurements alone using *t*-test, we found no significant differences between Mi.III and non-Mi.III (Table 5).

We also found that a roughly direct correlation between venous CO₂ and plasma HCO₃⁻ levels (Fig. 5, top). This plot shows that despite the considerable variability in blood CO₂/HCO₃⁻ levels among individuals, blood CO₂ levels were indiscriminately directly related to blood HCO₃⁻ levels. To decipher the data, we plotted pre-exercise and post-exercise data points of all subjects again using vector representation (Fig. 5). Each vector composes of the pre-exercise and post-exercise measurements for a single participant. The beginning of a vector is one’s pre-exercise value, and the arrowhead is his post-exercise value. The length of a vector indicates the magnitude of change, and its directionality indicates how relevant or irrelevant the 2 parameters (represented by x- and y-axes) are.

From vector analyses (Fig. 5, bottom), we found that majority of the non-Mi.III subjects showed reduced levels of blood CO₂ and HCO₃⁻ following exercise. In comparison, half of the Mi.III subjects showed small increases of blood CO₂, and the rest of the Mi.III subjects showed small decreases of blood CO₂ after exercise. The magnitudes of blood CO₂ changes among Mi.III subjects were generally smaller, compared to that in non-Mi.III (Fig. 5, bottom). The findings by vector representation supports the

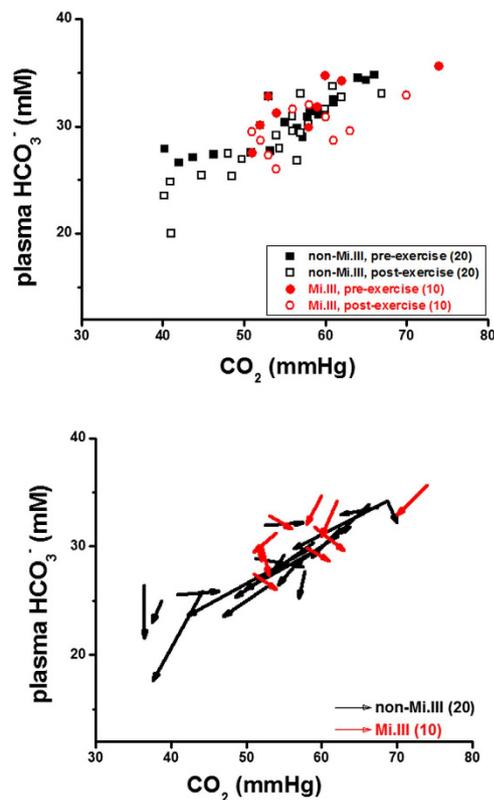


Figure 5. Mi.III and non-Mi.III people exhibited distinguishable blood CO₂ responses following the step test. Top: A roughly direct correlation between venous CO₂ and HCO₃⁻ levels in Mi.III and non-Mi.III subjects alike. The concentrations of venous CO₂ and HCO₃⁻ immediately before 3-minute step test (shown in solid symbols) and after the test (empty symbols) were plotted for individual Mi.III (red symbols) and non-Mi.III subjects (black). Bottom: To further dissect the changes of venous CO₂ and HCO₃⁻, as well as their correlations, for each subject, his pre-exercise and post-exercise data points were connected and represented by a vector. Almost all the subjects, Mi.III and non-Mi.III alike, showed reduced HCO₃⁻ levels after exercise. But noticeably, up to 70% of the non-Mi.III subjects had decreased blood CO₂ levels after exercise, and only 40% of the Mi.III subjects showed slight decreases of blood CO₂ following exercise. 50% of the Mi.III subjects showed slightly higher blood CO₂ after exercise. The numbers of the test subjects per group are specified in parentheses.

ANCOVA results (Table 5): exercise-induced venous CO₂ responses were significantly different between Mi.III and non-Mi.III.

The results thus suggest that blood CO₂/HCO₃⁻ homeostasis in Mi.III+ versus non-Mi.III people were distinguishable. Almost all the subjects showed slight decreases of plasma HCO₃⁻ following exercise (Fig. 5 & Table 5). Though CO₂ production increased during exercise, experimentally we and others observed reduced blood CO₂ immediately after exercise in most non-Mi.III subjects³¹. It has been suggested that exercise-induced hyperventilation decreases body CO₂ stores, in which bicarbonate is a main constituent^{31,32}. This explains the reduction of blood CO₂ and blood bicarbonate that we observed after the moderate exercise test (Fig. 5). Bicarbonate is also utilized to buffer excessive acidic metabolites generated during exercise, which would decrease the content of plasma HCO₃⁻ following exercise, too³³.

Conceptually, Mi.III RBCs express more AE1 on the cell surface than non-Mi.III RBCs (Fig. 1)¹⁸, and therefore Mi.III cells are expected to facilitate blood CO₂/HCO₃⁻ equilibrium more efficiently. Implied from our results (Fig. 5B), Mi.III expression might somehow affect blood CO₂ sensing. Blood CO₂ levels are primarily set by the respiratory center in the CNS³³. Because Mi.III expression allows for faster reach of blood HCO₃⁻ homeostasis and HCO₃⁻/CO₂ conversion, the respiratory center in Mi.III+ people would need to cope with faster changes of blood CO₂ levels at times. From what we found experimentally—the different venous P_{CO₂} responses post-exercise between Mi.III and non-Mi.III (Fig. 5B & Table 5), conceivably the respiratory center in Mi.III+ subjects could have higher tolerance for blood P_{CO₂}. This also explains, at least in part, why our healthy Mi.III subjects generally had slightly higher blood pressure than the non-Mi.III subjects (Table 1).

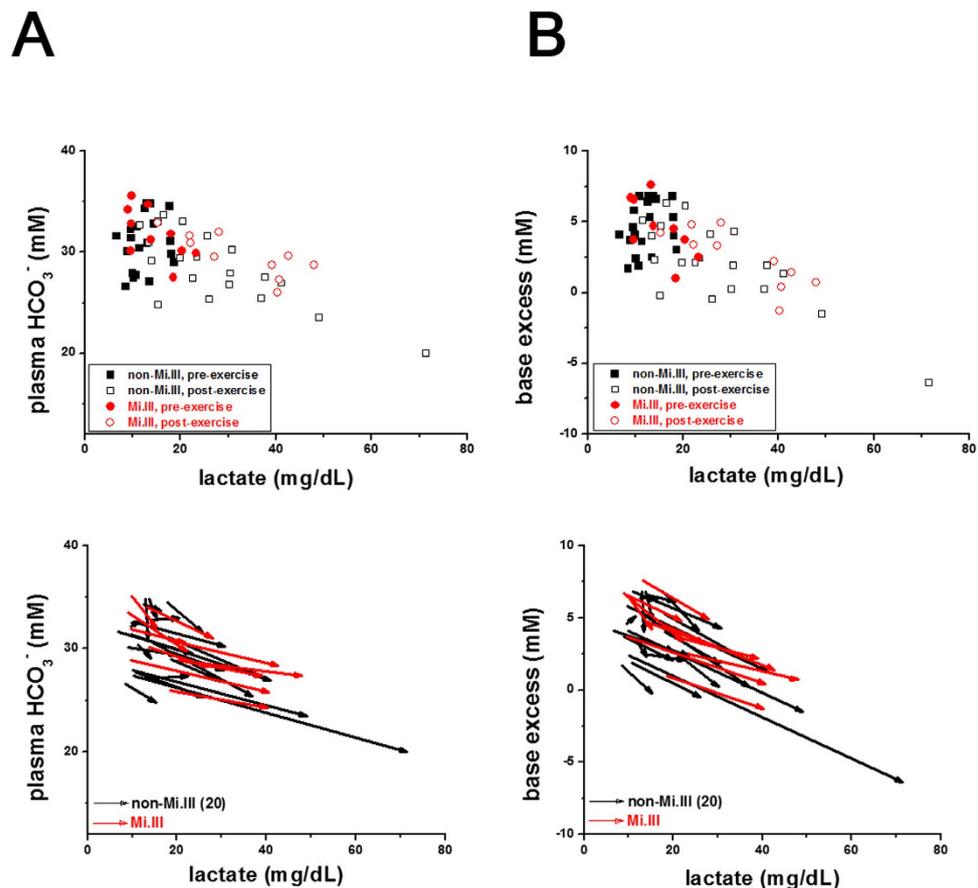


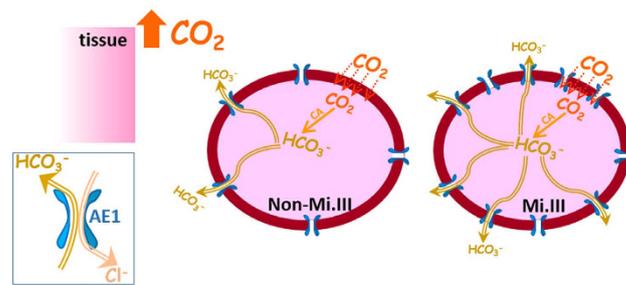
Figure 6. Mi.III and non-Mi.III people showed similar degrees of lactate production and base buffering following the exercise challenge. (A) roughly inverse correlation between plasma lactate and bicarbonate levels for all subjects before and after exercise. (B) A similar, inverse correlation between plasma lactate and base excess. Top: The concentrations immediately before 3-minute exercise (shown in solid symbols) and after exercise (in empty symbols) were plotted for individual Mi.III (red) and non-Mi.III (black) subjects. Bottom: The pre-exercise and the post-exercise data points per subject were connected and represented by a vector.

Similar degrees of anaerobic respiration induced by moderate exercise. With the moderate exercise test, we observed very small accumulation of plasma lactate in virtually all the test subjects (Fig. 6 & Table 5). Blood lactate begins to accumulate when tissue oxygen is low and insufficient to provide energy for muscle contraction. Lactate acid is generated from pyruvate during anaerobic glycolysis to release anaerobic energy, and is a major metabolite of anaerobic respiration. Overproduction of lactic acid in muscle tissues (e.g. from strenuous exercise) may lower blood pH, which would then require buffering by metabolites like phosphocreatine, bicarbonate, and proteins³². From the vector analyses (Fig. 6), all the subjects, Mi.III and non-Mi.III alike, exhibited reduced plasma bicarbonate (or base excess) with increased lactate post-exercise. Statistical analyses using either *t*-test or ANCOVA found no significant differences in the lactate levels between Mi.III and non-Mi.III (Table 5). Thus, Mi.III expression does not seem to affect anaerobic respiration (Fig. 6).

Discussion

Mi.III is the second most important erythrocyte blood type following ABO in the fields of transfusion medicine in Taiwan^{5,7}. Mi.III-encoded protein, GPMur, structurally is a hybrid of glycophorin B and glycophorin A; it functionally resembles that of glycophorin A in facilitating band 3 expression on the erythrocyte membrane^{18,25}. Higher expression of band 3 on Mi.III erythrocytes enlarges the capacity of HCO_3^- permeation across the red cell membrane¹⁸. Since band 3 is the rate-limiting factor for blood CO_2 metabolism¹², Mi.III+ people are expected to have more efficient CO_2 respiration. Here, we showed experimentally that Mi.III+ subjects exhibited faster CO_2 respiration and clearance immediately following a physical challenge (Figs. 2–4). Exercise-induced venous CO_2 responses were also distinguishable between Mi.III and non-Mi.III (Table 5 & Fig. 5). In contrast, Mi.III phenotype does not affect either aerobic parameters (i.e. heart rates and SpO_2) (Tables 3,4) or anaerobic respiration (Table 5 & Fig. 6).

A



B

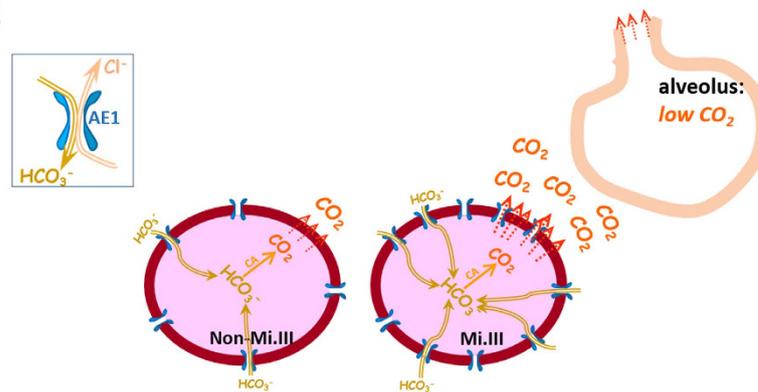


Figure 7. A proposed model on how CO₂ expiration could be faster in people bearing the Mi.III blood type than those lacking this phenotype. (A) During exercise, there is increasing CO₂ output, along with an increasing demand for ATP expenditure from contracting muscles. CO₂ produced by muscle tissues enters erythrocytes primarily through passive diffusion (shown in red dotted arrows). Erythrocytes contain abundant carbonic anhydrase, which functions to greatly accelerate the chemical conversion between CO₂ and HCO₃⁻. Inside circulating red cells in this vicinity, CO₂ is rapidly converted to HCO₃⁻ by carbonic anhydrase. Much HCO₃⁻ produced is passively transported out of RBCs via AE1 (shown as the blue gates embedded on the cell membrane). For every bicarbonate ion moving out of RBCs through AE1, a Cl⁻ ion is transported into RBCs to maintain electroneutrality across the cell membrane. The direction and magnitude of anion transport via AE1 follow the Donnan equilibrium, and thus this bi-directional anion transport does not consume energy. Mi.III RBCs express significantly more AE1 molecules on the membrane, allowing more efficient export of bicarbonate and faster reach of HCO₃⁻ homeostasis across the cell membrane. (B) P_{CO₂} drops to below 40 mmHg in the lungs. The P_{CO₂} differences between lung alveoli and capillaries drive CO₂ expiration. As most blood CO₂ is present in the form of HCO₃⁻, there is tremendous driving force for HCO₃⁻ to be converted into CO₂ in the lungs. Notably, the enzymatic activity of carbonic anhydrase to convert HCO₃⁻ into CO₂ is much faster than the rate of bicarbonate transport through AE1. Larger bicarbonate influxes into Mi.III RBCs that express more AE1, and consequently faster CO₂ production and expiration are expected from Mi.III+ individuals. The directionality of HCO₃⁻/Cl⁻ fluxes through AE1 reverse from tissues to lungs, which is primarily driven by the changes of HCO₃⁻ concentration gradients across the red cell membrane (as shown left in boxes). The diagram here is intended to illustrate the mechanism of how Mi.III expression could impact physiologic CO₂ respiration, and is not drawn to scale.

To put in context with our current understanding of respiratory physiology, we propose a model to illustrate how CO₂ expiration could be expedited especially in Mi.III+ people following a physical challenge (Fig. 7). In lung alveoli (P_{CO₂} < 40 mmHg), the driving force to convert blood bicarbonate to CO₂ is substantial. Since this conversion mainly takes place inside red cells, with larger bicarbonate influx capacities, Mi.III+RBCs in the lungs are expected to produce CO₂ faster than non-Mi.III erythrocytes. We indeed observed faster CO₂ expiration in people bearing the Mi.III blood type (Figs. 2,3). As

Mi.III+RBCs circulate to the tissue vicinities, they are expected to facilitate blood $\text{CO}_2/\text{HCO}_3^-$ homeostasis more efficiently than non-Mi.III cells, because of their higher AE1 expression (Fig. 7, top).

CO_2 accumulation increases breathing discomfort and reduces one's capability to endure physically⁹. Expedited CO_2 expiration reduces the rate or the degree of CO_2 accumulation in the body. Gorman *et al.* in their human study using inspiratory resistive loading found that lessening CO_2 accumulation through ventilation prolongs endurance time⁹. Conceivably, when physically challenged, Mi.III+ people with more efficient capabilities of CO_2 respiration are expected to suffer less from CO_2 accumulation or hypercapnia and be more capable of enduring physical stresses. Intriguingly, Ami tribespeople (88% with the Mi.III blood type) historically were recognized by the Japanese colonial government in Taiwan (1895–1945) to outperform other local ethnic groups in forced heavy labor^{10,11,26}. One of the main reasons that the Japanese ruling officials in Taiwan at that time favored Ami tribespeople for labor-intensive tasks was Ami's superior physical endurance, according to Yu-Chi Lai, a contemporary historian and anthropologist ethnic of Ami³⁴.

To probe into the relations between Mi.III expression and physical endurance, we recently surveyed elite athlete students at the National Taiwan Sport University who had won medals in regional and/or national sports competitions, and found that 22% of them (16/72) have the Mi.III blood type. Moreover, up to a third of the track and field athletes (9/28) are Mi.III+. Notably, not all these Mi.III+ elite athletes are ethnically Ami. About 11% of the non-Ami elite athletes have the Mi.III phenotype, which is at least double the frequency of Mi.III in the general Taiwanese population (2–6%)^{5,7}. The Mi.III phenotype therefore might be associated with athleticism, at least in part through its support of physiologic CO_2 respiration.

The $\text{HCO}_3^-/\text{Cl}^-$ exchange capacity (permeability) of Mi.III+RBCs is expandable upon high $\text{HCO}_3^-/\text{CO}_2$ stimulation¹⁸. People generate a lot of $\text{HCO}_3^-/\text{CO}_2$ when they are physically stressed (e.g. during rigorous exercise). Our results here indicate that Mi.III expression in erythrocytes facilitate physiologic CO_2 clearance. From this aspect, Mi.III-encoded GP.Mur protein could be perceived as a natural “de-stressor” through its support of band 3 expression. Expression of the Mi.III blood type is thus expected to be beneficial to one's health.

Materials & Methods

This human study aimed to test if GP.Mur phenotype could affect physiologic respiration through its support of band 3 expression.

Ethics Statement. The study was carried out in accordance with the principles of the Declaration of Helsinki, and was approved by the Institutional Review Board (IRB) of Taiwan Mackay Memorial Hospital (MMH) (MMH-IRB registration number: 11MMHIS038). Written informed consent was obtained from all subjects.

Study Design. Local adult subjects between 18–50 years of age were recruited at the three branches of MMHs in Taipei, Tamsui, and Taitung. To minimize interferences from ageing-related pulmonary function decline, people over 50 years old were refrained from participating in this study. The human trial included: (1) a questionnaire survey and initial physical assessments; (2) blood sample studies; (3) a 3-minute, step exercise test that accompanied respiratory physiological measurements, and/or blood tests. A quick prescreening of Mi.III was imposed to ensure that a high percentage of the test subjects have this special blood type. Prior to blood sample collection and the exercise test, each participant was first asked to fill out a questionnaire that surveys one's lifestyle and health conditions. We also conducted initial physical assessments to exclude people who were unfit for the exercise test, i.e. those who were hypertensive (diastolic blood pressure >90 mmHg or/and systolic blood pressure >140 mmHg), or/and whose quiet heart rates were above 100.

Blood tests and RBC phenotyping. Blood tests for all subjects participating in the trial included complete blood count (CBC), erythrocyte phenotyping including Mi.III, and a quantitative assessment of erythrocyte band 3 protein. The Mi.III RBC phenotype was serologically determined with anti-Mur, anti-Hil, anti-Anek, and anti-Mi^a antisera, each antiserum targeting a distinct epitope on GP.Mur protein. Mi.III phenotype was further confirmed by direct blood PCR, as described previously³⁵. Additionally, venous CO_2 , bicarbonate, pH, base excess, and plasma lactate were tested in some of the male subjects who volunteered to have their blood withdrawn immediately before and after the 3-minute stepping test.

Assessment of erythrocyte band 3 levels by DIDS labeling. RBC ghosts (membranes) were made by hypotonic rupture, followed by repeated washes to remove hemoglobin¹⁸. For each sample, RBC ghosts were mixed with an equal volume of 50 μM DIDS (Sigma-Aldrich, St. Louis, MO, USA), and incubated at 37°C for 30 minutes³⁶. The fluorescent compound DIDS binds specifically to lysing-539 of band 3²⁴. DIDS unbound to erythrocyte band 3 was then removed by PBS wash. DIDS labeling of band 3 was quantitated per sample by fluorescence emission using Varioskan™ Flash Multimode Reader (Thermo Scientific, Waltham, MA, USA) at the emission wavelength of 450 nm (excitation at 350 nm). After fluorescence background subtraction, each DIDS emission reading was normalized with respect to

the averaged value of all the Mi.III-negative samples, and expressed in percentile (the average of non-Mi.III data = 100%).

The 3-minute step test and physiological measurements. The standardized 3-minute step test designed by the Taiwan National Council on Physical Fitness & Sports is a modification of the original Harvard test³⁷. Like the Harvard step test, this standardized step test is to evaluate one's cardiorespiratory fitness levels. Slightly different from the Harvard test or the YMCA step test²⁷, the standardized step test used in Taiwan asks test subjects to step onto a 35-cm high sturdy bench at the rate of 24 steps (96 beats) per minute for continuous 3 minutes³⁷. Heart rates were measured immediately following the test, and were used to assess one's cardiorespiratory endurance index (CREI), as follows:

$$\text{CREI (\%)} = \frac{(\text{stepduration}) \times 100}{(\text{HR1} + \text{HR2} + \text{HR3})}$$

where step duration (in seconds) is 180 if the 3-minute test is completed. HR1, HR2, and HR3 refer to heart rates (beats/min) at the 1st, 2nd, and the 3rd minute immediately after the step test.

The Department of Physical Education under Taiwan Ministry of Education conducts such standardized fitness tests annually on at least tens of thousands of people from 6–65 years of age. These test results were compiled in the norm charts of cardiorespiratory endurance indices for Taiwan populations²⁸. In our study, besides heart rates, end-tidal CO₂ (EtCO₂), blood O₂ saturation (SpO₂), and respiration rates before and right after the step test were also measured using a portable oximetry instrument (MD-667P; COMDEK Inc., New Taipei City, Taiwan).

ΔEtCO₂/min Refers to the minute-to minute change of EtCO₂ following the step test. ΔEtCO₂/min at the Nth minute post-exercise was calculated by subtracting EtCO₂ at the Nth minute from EtCO₂ at the previous or the (N-1)th minute post-exercise. Thus, ΔEtCO₂/min represents the rate change of CO₂ expiration.

Statistical analyses. For most data, unless specified otherwise, the levels of significance (*p*) were determined by two-sample *t*-test. For analyses of blood test results obtained immediately before and after 3-minute step, separate analyses of covariance (ANCOVAs) were performed to examine the effects of Mi.III phenotype on each parameter. In ANCOVA, the fixed factor was the expression or the absence of Mi.III phenotype, pre-exercise measurements were used as covariate, and post-exercise measurements as dependable variables. A *p* value less than 0.05 was deemed significant.

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Author Contributions

K.H. and M.S.K. conceived the projects. M.S.K., Y.C.C., H.C.C., C.H.L., H.J.L. and T.H.Y. conducted the human trial. C.C.Y., H.J.L. and T.Y.L. carried out lab experiments and analyzed the data. K.H. wrote the paper.

Additional Information

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