Increased Blood Serum Levels of Proinflammatory Cytokines Accompany Increased Fatigue in T-6A Texan II Instructor Pilots

21-24 March 2023

RAMS/NATO STO HFM-350 Aerospace Medical Technical Course

Garmisch-Partenkirchen, Germany

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Disclosures
We have no actual or potential conflicts of interest to disclose in relation to this presentation.

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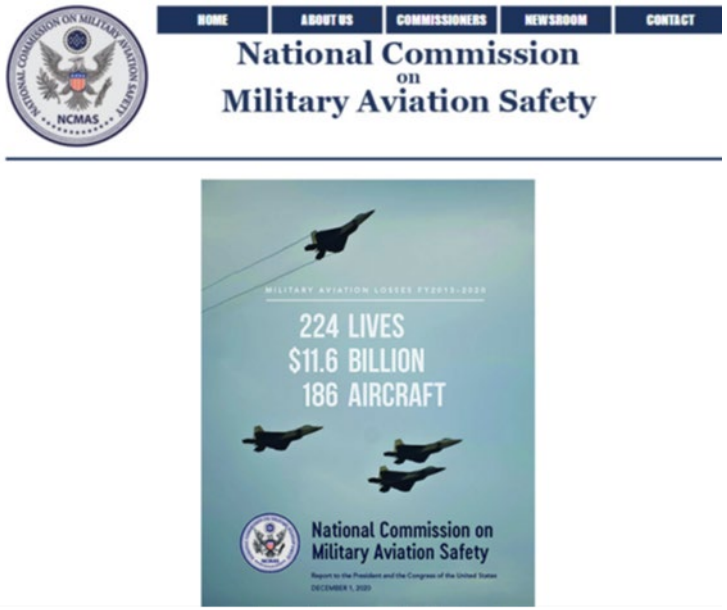
Source of Support
Department of Defense — USAF: 711th Human Performance Wing *"Establishing biomarkers of post- sortie cognitive fatigue"*

Human Research Protections/IRB Statement
The study protocol was approved by the Naval Medical Research Unit Dayton Institutional Review Board in compliance with all applicable federal regulations governing the protection of human subjects. A reliant agreement was arranged with the Case Western Reserve University Institutional Review Board.

All photographs and images are courtesy of the Decker lab

Cognitive Fatigue

- A constant threat to human performance and aviator safety, is the “likely cause of the next mishap”
  - *National Commission on Military Aviation Safety: Report to the President and Congress*



Preventing that mishap is hampered by an absence of quantitative physiologic biomarkers that correspond to and predict increasing levels of <u>fatigue</u>.

Cody, R.A., Healing, R.F., Donnelly, S.C., Johns, R.E., Geren, P., Kern, D.R., and Hagin, J.W. (2020). National Commission on Military Aviation Safety. Report to the President and the Congress of the United States, 1-143.

Fatigue vs. Sleep Loss

- Sleep loss is traditionally considered the primary cause of fatigue in aviators

ICAO-International Commercial Aviation Organization. (2013). Excerpts of fatigue management-related provisions from Annex 6 to the Convention on International Civil Aviation. International Standards and Recommended Practices - Operation of Aircraft Part I: International Commercial Air Transport - Aeroplanes.

- ***Both fatigue and sleepiness have been shown to emerge following increased systemic and central nervous system levels of proinflammatory cytokines in multiple clinical populations.***
- No studies have been performed in tactical aviators, who are healthy and fit individuals, to determine if they also experience increased levels of proinflammatory cytokines when fatigued

Raison, C.L., Lin, J.-M.S., and Reeves, W.C. (2009). Association of peripheral inflammatory markers with chronic fatigue in a population-based sample. Brain. Behav. Immun. 23, 327-337. doi: 10.1016/j.bbi.2008.11.005

Dantzer, R., Heijnen, C.J., Kavelaars, A., Laye, S., and Capuron, L. (2014). The neuroimmune basis of fatigue. Trends. Neurosci. 37, 3 9-46. doi: 10.1016/j.tins.2013.10.003

Our Hypothesis

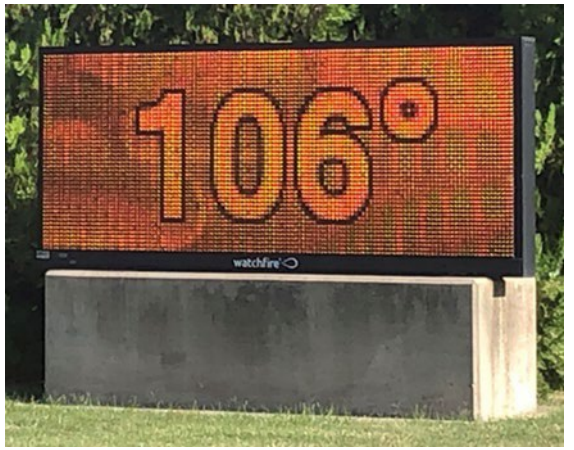
Recurrent exposure to the physical challenges of high-performance aviation → continual synthesis and release of proinflammatory cytokines. Upon reaching a critical threshold, cognitive fatigue will emerge.

Hyperoxia

4G dive

Hypergravity

Visuospatial

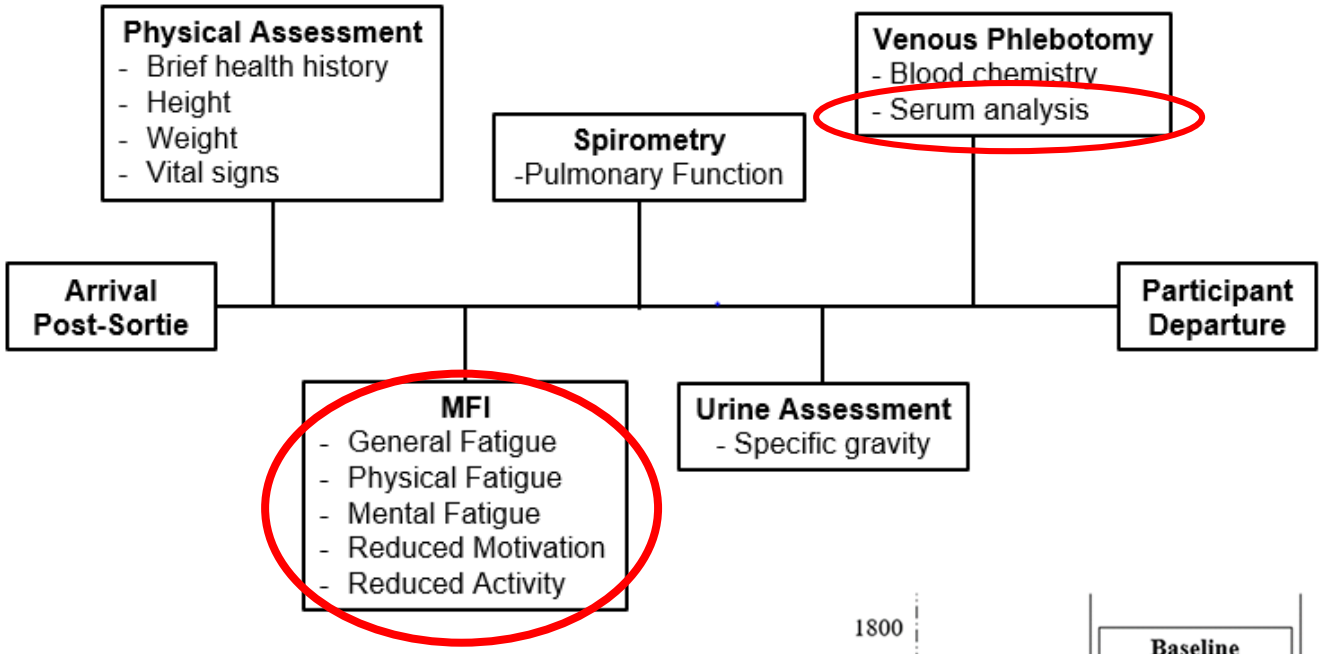


Thermal stress

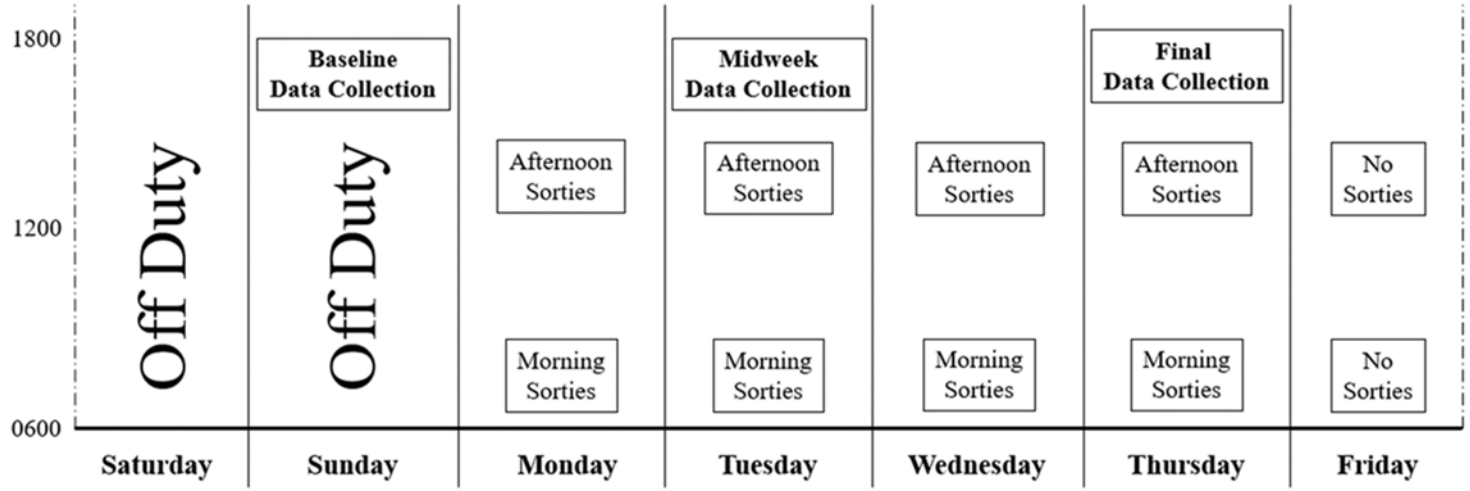
The Study Cohort

- Study participants were T-6A Texan II Instructor Pilots who were scheduled to fly at least two flights during the week of data collection.
- Data were collected on three separate days across the week-long flying schedule.

What we measured



When we measured it



Results

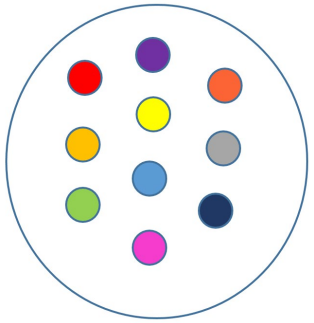
Sample Demographics (N=22)

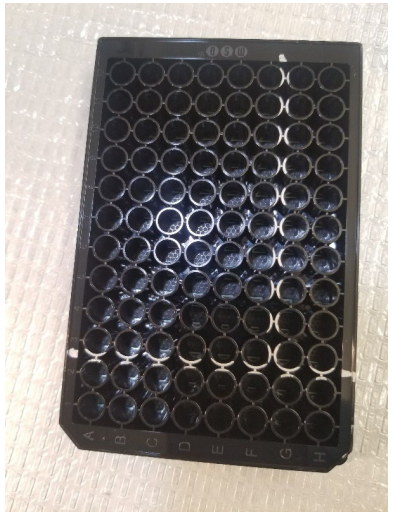
Sex	Age (years) M ± SD *(Range)*	BMI (kg/m^2) M ± SD *(Range)*
Males **n = 20**	**37.95 ± 4.73** ***(29-47)***	**26.63 ± 3.15** ***(21.92-32.63)***
Females **n = 2**	**41.00 ± 0.00** ***(41-41)***	**24.38 ± 1.61** ***(23.24-25.52)***
p-value	**0.21**	**0.36**

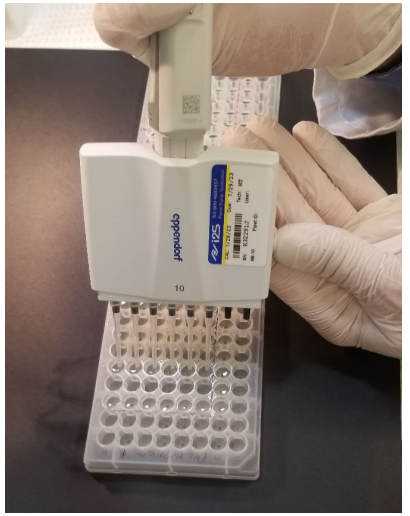
No age or BMI differences between males and females
(p-values from Wilcoxon rank sum test)

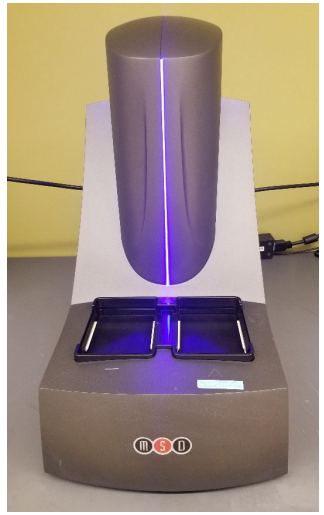
Blood Serum Analysis: Electrochemiluminescence

- We analyzed serum using multi-array technology, which provided the capability to simultaneously measure 4-10 analytes in the same well. We used the following preconfigured 96 well plates: Proinflammatory, Chemokine, Cytokine, Angiogenesis, Vascular Injury, and Obesity.
- Serum was placed into each well where analytes of interest were captured by the antibody-coated carbon electrodes and then detected by an analyte-specific ruthenium-conjugated secondary antibody.
- When the secondary antibody was electrochemically stimulated, it emitted light with the intensity determined by the concentration of the analyte within the sample.









BLUF

- **Baseline** measures of serum analytes (Sunday) were comparable to currently available reference values.
- By the conclusion of the second day of the flying schedule (Tuesday), serum levels of multiple analytes diverged from baseline values, **with 20 (of 42) becoming significantly different** by the fourth day of flying.
  - [Serial measurement of serum analytes we measured are stable in healthy persons not exposed to extreme physical activity]

- *Biancotto, A., Wank, A., Perl, S., Cook, W., Olnes, M.J., Dagur, P.K., Fuchs, J.C., Langweiler, M., Wang, E., and McCoy, J.P. (2013). Baseline levels and temporal stability of 27 multiplexed serum cytokine concentrations in healthy subjects. PLoS. One. 8, e76091.*
- *Wu, D., Dinh, T.L., Bausk, B.P., and Walt, D.R. (2017). Long-term measurements of human inflammatory cytokines reveal complex baseline variations between individuals. Am. J. Pathol. 187, 2620-2626.*

Proinflammatory Panel (pg/mL) N=22

	Baseline (M ± SD) *(range)*	Midweek (M ± SD) *(range)*	Final (M ± SD) *(range)*	*p*-value (Baseline to Final)
IL-4	0.05 ± 0.06 *(0.005-0.26)*	0.05 ± 0.07 *(0.003-0.25)*	0.07+ ± 0.05 *(0.02-0.22)*	0.001*
IL-8	8.46 ± 3.63 *(4.38-16.42)*	10.97 ± 5.01 *(4.24-20.64)*	11.00 ± 3.92 *(5.99-20.76)*	0.003*
IL-10	0.41 ± 0.36 *(0.12-1.59)*	0.38 ± 0.35 *(0.09-1.59)*	0.26 ± 0.20 *(0.09-1.03)*	0.018

*Indicates significance following Bonferroni-Holm correction

No change in interleukin 1 Beta (IL-1β), interleukin 2 (IL-2), interleukin 6 (IL-6), interleukin 13 (IL-13), tumor necrosis factor alpha (TNF-α), interferon-gamma (IFN-γ)

Chemokine Panel (pg/mL) N=22

	Baseline (M ± SD) *(range)*	Midweek (M ± SD) *(range)*	Final (M ± SD) *(range)*	*p*-value (Baseline to Final)
MCP-1	127.42 ± 45.02 *(55.21-267.11)*	162.81 ± 68.35 *(59.19-311.9)*	175.33 ± 70.61 *(55.83-336.39)*	0.002
MCP-4	41.86 ± 37.65 *(9.80-184.13)*	50.57 ± 35.05 *(7.13-163.06)*	54.97 ± 34.24 *(16.14-163.30)*	0.008
Eotaxin-3	6.75 ± 5.36 *(1.40-22.88)*	10.32 ± 6.29 *(0.24-23.79)*	13.44 ± 7.17 *(3.06-35.44)*	0.002*
TARC	73.58 ± 54.21 *(16.50-230.36)*	101.55 ± 70.12 *(23.27-293.91)*	110.64 ± 50.26 *(21.51-229.57)*	0.009
MIP-1α	8.99 ± 4.65 *(3.61-24.27)*	11.55 ± 9.16 *(2.21-41.03)*	10.41 ± 2.76 *(4.08-15.39)*	0.020
MIP-1β	68.77 ± 23.70 *(37.91-136.96)*	82.79 ± 37.19 *(36.00-167.29)*	88.60 ± 28.10 *(45.13-159.98)*	0.002*

*Indicates significance following Bonferroni-Holm correction

No change in eotaxin, macrophage-derived chemokine (MDC), interferon-gamma inducible protein of 10 kDa (IP-10).

Cytokine Panel (pg/mL) N=22

	Baseline (M ± SD) *(range)*	Midweek (M ± SD) *(range)*	Final (M ± SD) *(range)*	*p*-value (Baseline to Final)
IL-7	10.90 ± 5.81 *(3.80-27.46)*	14.90 ± 7.81 *(4.04-36.02)*	20.28 ± 8.11 *(7.40-34.56)*	<0.0001*
IL-12	71.88 ± 31.33 *(36.46-173.69)*	74.22 ± 39.84 *(27.36-191.47)*	89.59 ± 42.94 *(39.91-237.60)*	0.0001*
IL-15	2.06 ± 0.30 *(1.66-2.61)*	1.94 ± 0.46 *(1.12-2.72)*	2.27 ± 0.34 *(1.76-3.04)*	0.018
IL-17A	1.09 ± 0.35 *(0.42-1.83)*	1.24 ± 0.60 *(0.45-2.72)*	1.74 ± 0.52 *(1.11-3.34)*	<0.0001*
VEGF-A	43.25 ± 27.34 *(8.58-134.42)*	70.46 ± 63.03 *(9.27-263.24)*	118.28 ± 63.05 *(34.82-276.96)*	<0.0001*

*Indicates significance following Bonferroni-Holm correction

No change in interleukin 1 alpha (IL-1α), interleukin 5 (IL-5), interleukin 16 (IL-16), tumor necrosis factor beta (TNF-β).

Angiogenesis Panel (pg/mL) N=22

	Baseline (M ± SD) *(range)*	Midweek (M ± SD) *(range)*	Final (M ± SD) *(range)*	*p*-value (Baseline to Final)
VEGF-D	1551.86 ± 405.37 *(1068.68-2709.09)*	1357.24 ± 388.18 *(834.71-2523.37)*	1148.91 ± 286.80 *(762.09-1961.71)*	<0.0001*
Tie-2	980.50 235.84 *(274.87-1363.72)*	900.91 253.11 *(296.72-1355.75)*	737.84-175.32 *(187.54-1046.33)*	<0.0001*

*Indicates significance following Bonferroni-Holm correction

No change in basic fibroblastic growth factor (bFGF), vascular endothelial growth factor C (VEGF-C), vascular endothelial growth factor receptor 1 (Flt-1), placental growth factor (PlGF).

Vascular Injury Panel (pg/mL) N=22

	Baseline (M ± SD) *(range)*	Midweek (M ± SD) *(range)*	Final (M ± SD) *(range)*	*p*-value (Baseline to Final)
VCAM-1	640629.35 ± 293150.58 *(322327.73-1261489.42)*	517489.62 ± 215525.34 *(238663.56-1067055.15)*	465626.35 ± 103173.38 *(326028.23-741180.39)*	0.006*

*Indicates significance following Bonferroni-Holm correction

No change in serum amyloid A (SAA), C-reactive protein (CRP), intercellular adhesion molecule-1 (ICAM-1).

Obesity Panel (pg/mL) N=22

	Baseline M ± SD *(range)*	Midweek M ± SD *(range)*	Final M ± SD *(range)*	*p*-value (Baseline to Final)
BDNF	17.72 ± 23.86 *(3.88-106.87)*	171.51 ± 222.87 *(4.30-673.10)*	372.95 ± 443.62 *(9.35-1727.51)*	<0.0001*
Leptin	7579.36 ± 6122.32 *(398.99-24226.97)*	7207.24 ± 5036.27 *(634.99-16857.55)*	6247.65 ± 5414.35 *(384.04-17973.04)*	0.015
Glucagon	24.57 ± 11.04 *(6.14-48.84)*	13.88 ± 9.57 *(2.29-43.04)*	10.83 ± 9.09 *(2.74-45.02)*	<0.0001*

*Indicates significance following Bonferroni-Holm correction

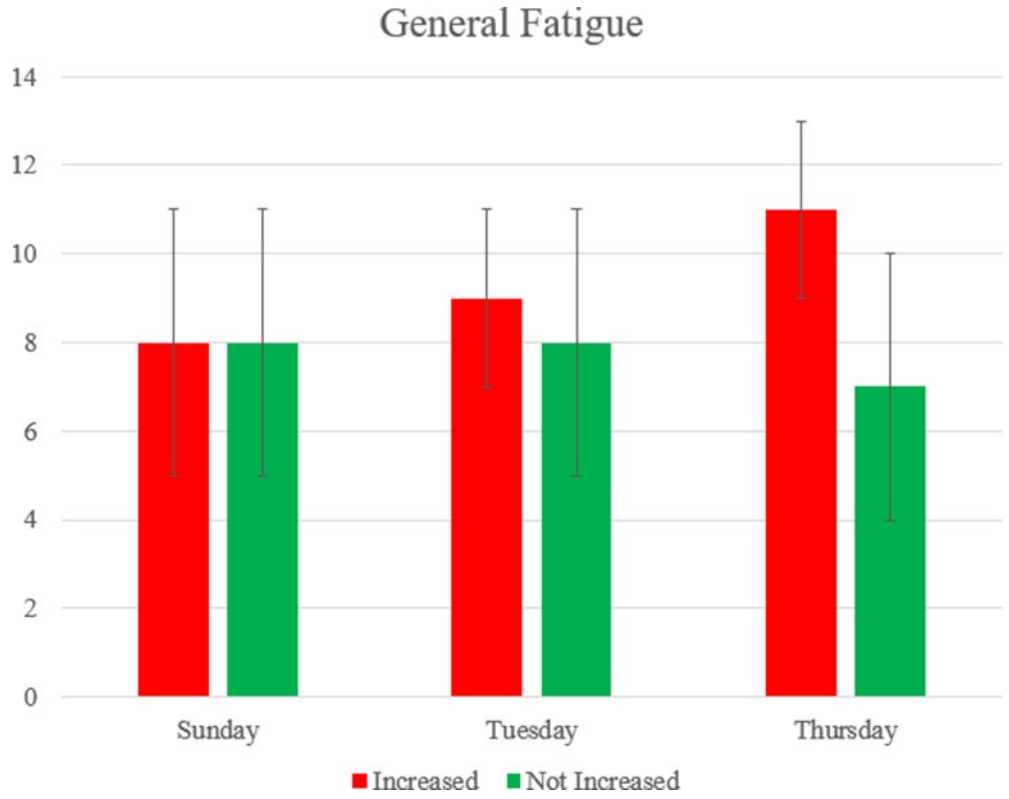
No change in ghrelin or fibroblast growth factor 21 (FGF-21).

In Summary…

- Urine Specific Gravity, Calculated Plasma Volume, Hematocrit, and Blood Chemistry values were stable across the work week
- **Twenty of 42 blood serum analytes significantly changed** across the study. Many values increased while others decreased.

Do the changes in serum analytes correspond to levels of fatigue?

- Upon examining fatigue scores, we observed that not all pilots reported increased fatigue across the week
- Participants were then categorized into two groups:
  - Final General Fatigue score higher than baseline (n=13)
  - Final General Fatigue score not higher than baseline (n=9)



Relationship between Fatigue and Serum Analytes

- We next assessed the relationship between fatigue scores and serum analytes.
  - A repeated measures linear mixed model with compound symmetric covariance structure analysis including fatigue group by time interaction was performed to compare values at each of the three data collection time points

Relationship between Fatigue and Serum Analytes

Analyte	Estimate (β) of General Fatigue Score†	SE	95% CI	*p*-value[a] Baseline to Final
Proinflammatory Panel				
*TNF α	0.1620	0.0745	(0.0059, 0.3180)	0.0427
Chemokine Panel				
MCP1	95.7499	21.2057	(51.3659, 140.1340)	0.0002
*MCP4	0.6250	0.1459	(0.3196, 0.9305)	0.0004
Eotaxin	55.3390	21.2115	(10.9429, 99.7352)	0.0173
*Eotaxin-3	0.5525	0.2455	(0.0387, 1.0662)	0.0364
TARC	49.9432	17.3052	(13.7229, 86.1634)	0.0095
MIP-1β	29.4478	9.7810	(8.9759, 49.9197)	0.0072
*MDC	0.2369	0.0864	(0.0561, 0.4178)	0.0130
Cytokine Panel				
IL15	0.2712	0.1204	(0.0192, 0.5231)	0.0363
*VEGF-A	0.4755	0.2164	(0.0226, 0.9284)	0.0406
Obesity Panel				
*BDNF	1.3501	0.5397	(0.2205, 2.4797)	0.0217

11 Serum Analytes were significantly associated with increased levels of General Fatigue

* Indicates log transformation prior to analysis

The majority of pilots did not feel “rested” BUT they were not “sleepy”

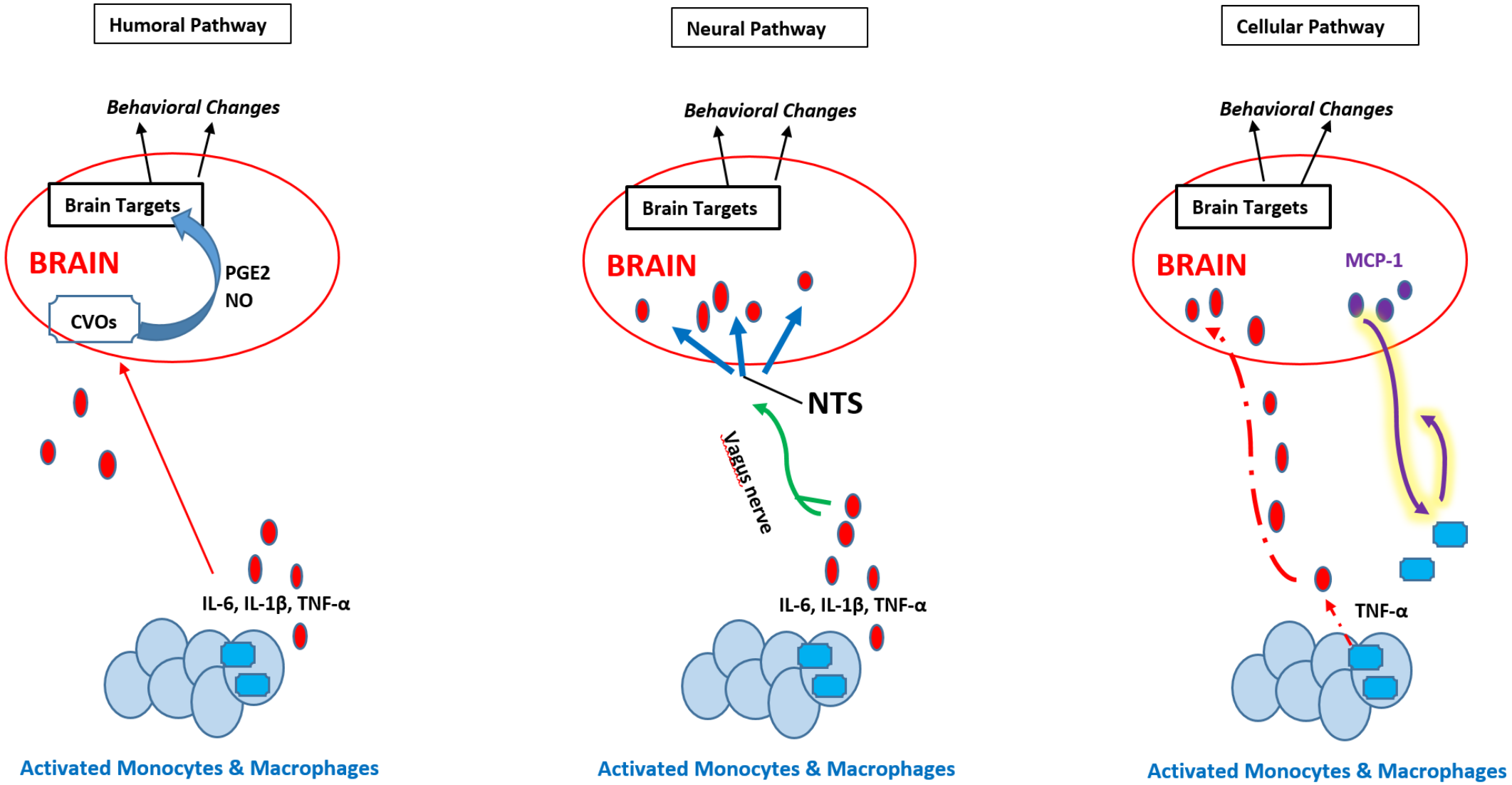
- Sleep duration (5-9.5 hours/night) was similar to that observed in the general population and longer on the weekend by ~ 1 hour, *p*=0.01
- ~ 7 of 22 pilots felt *“sleepy”* during the past 24 hrs. 15 of 22 were not *“sleepy”*

	Baseline	Midweek	Final
SLEEPY DURING THE DAY IN THE PAST 24 HOURS?			
Yes	6 *27.3%*	8 *36.4%*	7 *31.8%*
No	16 *72.7%*	14 *63.6%*	15 *68.2%*
DID YOU FEEL RESTED UPON AWAKENING THIS MORNING?			
Yes	11 *50%*	8 *36.4%*	7 *31.8%*
No	11 *50%*	14 *63.6%*	15 *68.2%*

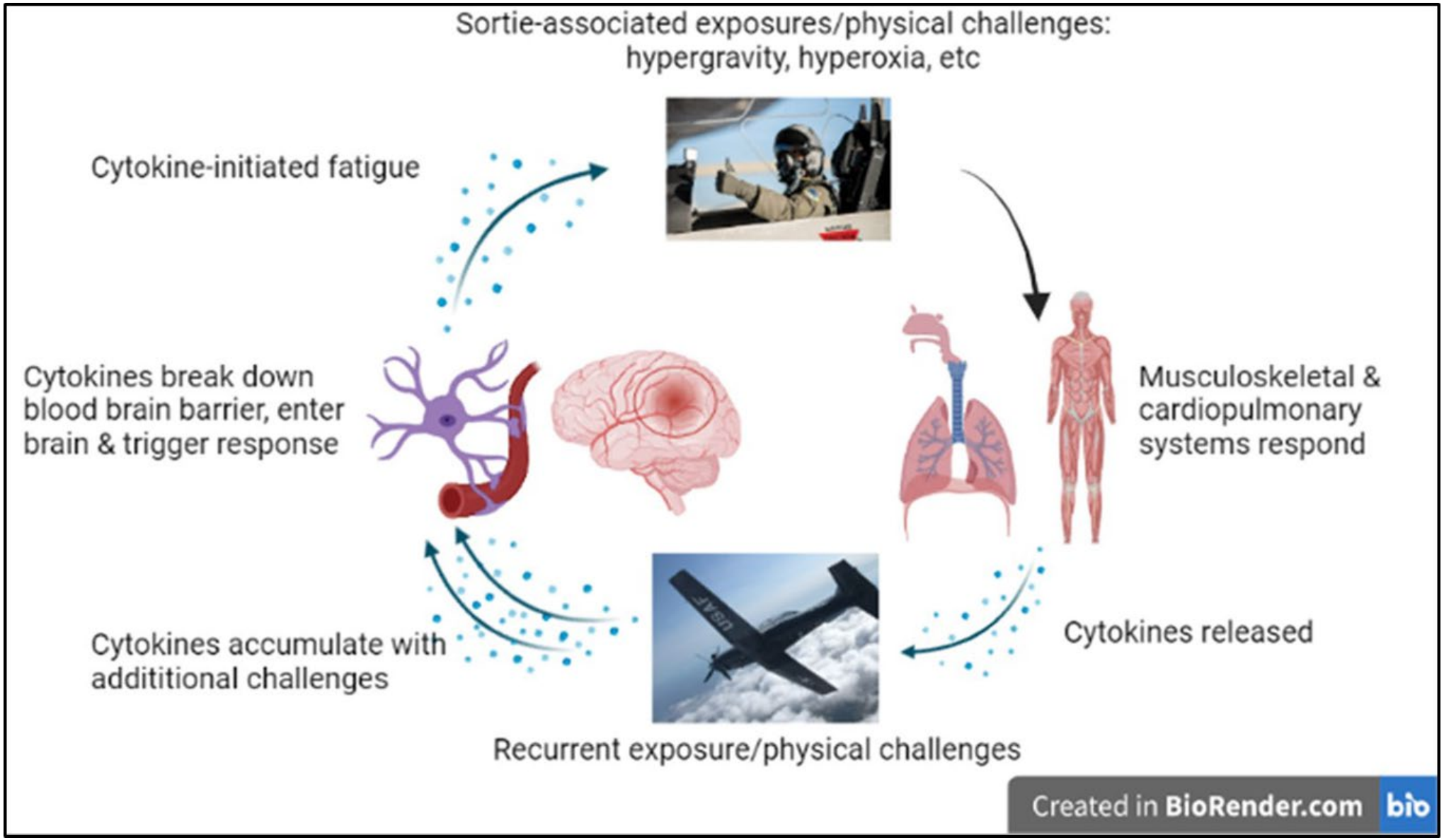
~ 11 of 22 pilots were not rested upon awakening (Baseline). That number increased to 15 of 22 by the study endpoint.

How might cytokines be inducing fatigue?

Models of Cytokine-Induced Fatigue



As described in: *Capuron, L., Miller, A.H. (2011). Immune system to brain signaling: Neuropsychopharmacological implications. Pharmacol Ther. 130, 226-238. doi: 10.1016/j.pharmthera.2011.01.014*


Sortie-associated exposures/physical challenges:
hypergravity, hyperoxia, etc
Cytokine-initiated fatigue
Cytokines break down
blood brain barrier, enter
brain & trigger response
Musculoskeletal &
cardiopulmonary
systems respond
Cytokines released
Cytokines accumulate with
addititional challenges
Recurrent exposure/physical challenges
Created in BioRender.com
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Supporting Flight Data

- This study was observational (not a controlled experiment)—not all subjects had equivalent exposures due to flying schedules
- Some important variables differed between the group that did and did not report increased fatigue
  - Biserial correlation analyses yielded significant positive correlations between onset of increased General Fatigue with the number of completed sorties ($r = 0.50$, $p = 0.017$) and total time spent executing sorties ($r = 0.45$, $p = 0.036$).
  - Spearman correlation analysis suggested that maximum +Gz was significantly associated with increased General Fatigue scores at the study endpoint (Thursday) ($r = 0.59$; $p = 0.008$).

Flight Information		
	Fatigue Scores Increased	**Fatigue Scores did not increase**
	M ± SD ***(range)***	**M ± SD** ***(range)***
Flights (# per week)	5.15 ± 1.28 *(3-7)*	4.00 ± 1.41 *(2-7)*
Flight Duration (minutes)	449.15 ± 93.68 *(293-570)*	358.33 ± 151.86 *(165-647)*
Maximum +Gz	5.27 ± 0.44 *(4.5-6.0)*	5.17 ± 0.61 *(4.0-6.0)*

Published in April 2022 in Frontiers in Physiology

frontiers | Frontiers in Physiology

ORIGINAL RESEARCH
published: 28 April 2022
doi: 10.3389/fphys.2022.876750

Increased Serum Levels of Proinflammatory Cytokines Are Accompanied by Fatigue in Military T-6A Texan II Instructor Pilots

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Specialty section:
This article was submitted to Environmental, Aviation and Space Physiology,

Tactical aviation imposes unprecedented physical challenges including repetitive exposure to hypergravity, hyperoxia, increased work of breathing, and profound cognitive workloads. Each stressor evokes outcomes ranging from musculoskeletal duress and atelectasis to physical and cognitive fatigue, the latter among the foremost threats to aviators. Whereas sleep loss is traditionally considered the primary cause of fatigue in aviators, converging experimental, observational, and medical studies have identified biochemical mechanisms promoting onset of fatigue. Those mechanisms, which fundamentally differ from sleep loss, revolve around increased proinflammatory cytokines, produced and released in response to tissue injury, chronic inflammatory disorders, allergens, or physical duress. This study's objective was to inform our understanding of potential relationships between serum levels of proinflammatory cytokines and onset of fatigue within a cohort of aviators who experience multiple high-performance sorties on a daily basis.

Thank you for your time and attention!

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