ASSESSING MCMV INFECTION AS A DRIVER OF CLINICAL FRAILTY IN AGING MICE

By

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With Honors in

Molecular and Cellular Biology

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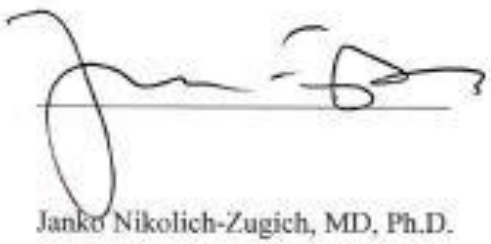
Approved by:



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Abstract

The topic of aging is widely discussed in the clinical setting, especially its little known distinction from frailty. Frailty, a syndrome, can be measured clinically using a set of parameters known as a Frailty Index (FI). Alongside the discussion of aging comes the topic of cytomegalovirus (CMV), a latent herpes virus that is contracted early and remains in the host until its death. This virus has been associated with many diseases with links to inflammatory problems, as CMV increases the inflammatory response. It is hypothesized that CMV also affects increased frailty in aging adults. To test this hypothesis, two groups (n=15) of mice, one infected with MCMV and one naive, were studied under a clinical frailty index for 13 months. We monitored the overall changes to the T cell compartments in the two groups by flow cytometric analysis of naïve and memory populations. Further, the size of the CMV-specific immune response was tracked by assessment of T cells specific for 3 viral proteins (M45, M38, and m139). These parameters were compared in individual mice against their increasing frailty index scores over the lifespan. We found no relationship between CMV infection, the magnitude of the CMV-specific T cell response, and frailty.

Introduction

Aging and Frailty

Aging and frailty, two related but separate topics, are rarely discussed colloquially to understand the differences between a natural process of prolonged life (aging), and as a syndrome (frailty). The concept of frailty as a syndrome, and not a natural product of aging, has evolved rapidly over the last few decades due to increased experience of geriatricians and is now clinically well recognizable [1]. While the method for evaluating frailty clinically is still being

explored, frailty can generally be characterized by increased vulnerability to stress due to decline in homeostasis, and dysregulation in multiple interrelated body systems [2]. These vulnerabilities ultimately lead to adverse health outcomes in the future, but how to recognize it clinically is still under debate. There are many schools of thought to approaching frailty and diagnosing it in aging adults, but two have emerged as the most reliable, repeatable, and testable. The frailty phenotype (also known as Fried's definition, or Cardiovascular Health Study (CHS) definition), is defined by five major categories of impairment: weakness as measured by decreased grip strength, slowed walking speed, low level of physical activity, exhaustion (self-reported) or low energy, and unintentional weight loss. Meeting three or more of these impairments categorizes the patient as frail, while meeting only one or two criteria is labeled prefrail [3]. The Rockwood approach is another approach to frailty that includes a more comprehensive approach. It takes an index, usually measuring upwards of 40 different physical parameters, and evaluates an individual based on a point system, usually 0-1. In order to consider a symptom to be a deficit in the frailty index (FI), it must be acquired (versus being born with it), age-related, associated with adverse outcomes, and the proportion of elderly adults that acquire the symptom should not be close to 100%, or else the deficit is uninformative [3]. Overall the differences between these two approaches are in how strict they are willing to categorize adults into a syndrome category. The Fried method is clear and concise, viewing frailty as a clinical syndrome. The Rockwood approach considers frailty to be a cumulative index and is variable, serving as a biological indicator of age instead of a chronological counter [2].

While these are great markers for clinical, visible signs of frailty, the body undergoes immunological changes and increased inflammation over the lifespan that plays a great role in frailty as well. Low-level inflammation has been found to be strongly associated with frailty,

consistent with the findings that inflammation is associated strongly with aging and chronic age-related diseases. This inflammation could be triggered many ways; a commonly studied stressor is the presence of cytomegalovirus, a persistent herpesvirus that stresses the immune system by causing CD8+ T cells to undergo prolonged activation and production of proinflammatory cytokines [4]. There are several molecular markers of chronic inflammation and immune activation in frailty. Elevated levels of IL-6 and TNF-α have been found in frail older adults, as well as increased levels of neopterin - a molecular marker for immune activation of monocytes and macrophages that arise independently of IL-6 levels, suggesting that immune system activation can also lead to chronic inflammation in the pathogenesis of frailty [3]. Other diseases, such as Alzheimer's, atherosclerosis, autoimmune disorders, and advancing age may also contribute to chronic inflammation that potentially leads to frailty [1]. While these causes are also being researched to either eradicate or alleviate symptoms, each of those cures would help a mere subset of the elderly in need. That is why attention has been turned to CMV and its effects on frailty, including the possibility of creating a vaccine for this virus.

CMV infection

CMV has been shown to cause serious consequences in fetuses and newborns as well. Among newborns, CMV is the leading cause of congenital infection in the developed world, and out of ~28,000 children born with congenital CMV infection in the US, ~150 die from the infection and >5,500 have permanent disabilities [11]. Because it is contracted through bodily fluid contact, pregnancy and CMV have caught the attention of many physicians. The CDC reports that over half of adults over 40 have contracted CMV [12], making the need for a vaccine and other preventative methods necessary in preventing not only congenital cases of CMV

infection and death, but also to reduce the possible long term effects CMV has on aging and frailty related diseases.

Persistent CMV infection, as previously mentioned, is associated with increased levels of pro-inflammatory biomarkers. The virus itself, however, is a latent herpes virus that drives naive CD8+ T cells into terminally differentiated states, and has been associated with older individuals having a harder time battling infections, including influenza [5]. CMV is never cleared from the body, and as a result has been linked to many chronic diseases with inflammatory components. Cardiovascular disease, cancer, cognitive decline, vascular dementia, and functional impairment are all examples of these [6]. Because the virus leaves no symptoms or clinically observable traces of infection in healthy individuals, it remains very difficult to treat and prevent [9]. In studies involving community-dwelling older women aged 70-79 years old, those with the largest CMV-directed immune response (as measured by CMV IgG levels, and reporting on those in the highest quartile of CMV IgG antibody concentration) had higher risks of 3-year incident frailty and 5-year mortality over those who were negative for CMV [9]. Persistent CMV infection has also been associated with prevalent frailty and significant multiplicative interaction with high levels of IL-6, leading the question to remain at how much CMV infection plays a role in frailty, and how much is from the byproduct of increased inflammation as a result of the infection [7].

When a new pathogen enters the body, it is recognized by naive T cells which then proliferate and differentiate into several types of effectors to combat the pathogen, such as cytotoxic T cells, and helper T cells which then carry out a cell-mediated response. At the peak of the infection, two populations are evident by cell surface phenotype: short lived effector cells (SLECs) and memory precursor effector cells (MPECs). After the initial infection is cleared, the majority of the short lived effector cells (SLECs) die via apoptosis. This acute infection can be

categorized, then, by high levels of SLECs. In contrast, the MPECs remain in the immune system. Upon reinfection, these cells will recognize previous pathogens and stimulate a faster, more aggressive immune response to a repeat pathogen to clear it from the body quicker. After the infection is cleared, or a persistent infection has resided for some time, a higher level of MPECs can be seen.

CMV infection has a distinct pattern of T cell response over the course of infection. T cells that will become stable memory T cells decline rapidly after acute infection and then persist at low frequencies. By using flow cytometry, the T cells that bind with CMV-specific antigens can be detected with MHC:peptide tetramers that are labeled with fluorophores. When the cells are then excited with a laser, different wavelengths of light will cause an emission of the different fluorophores, allowing them to fluoresce and indicate how many of the specific T cells are binding with the tetramers, and in general count the number of antigen-specific T cells that are responding. These CMV-specific T cells can then be interrogated for their MPEC or SLEC phenotype. In B6 mice infected with murine CMV (MCMV), the stable memory T cell pool is identified by T cells specific for the viral proteins M45 and M57 [8]. On the other hand, inflationary T cells increase in number after acute infection before stabilizing at a high frequency for the remainder of the infection (for life). In B6 mice infected with MCMV, populations specific for viral proteins encoded by m139, M38, and IE3 inflate dramatically over the 8–12 weeks post infection [8]. These cells will have high levels of short-lived effector cells that will run their course and apoptose [10].

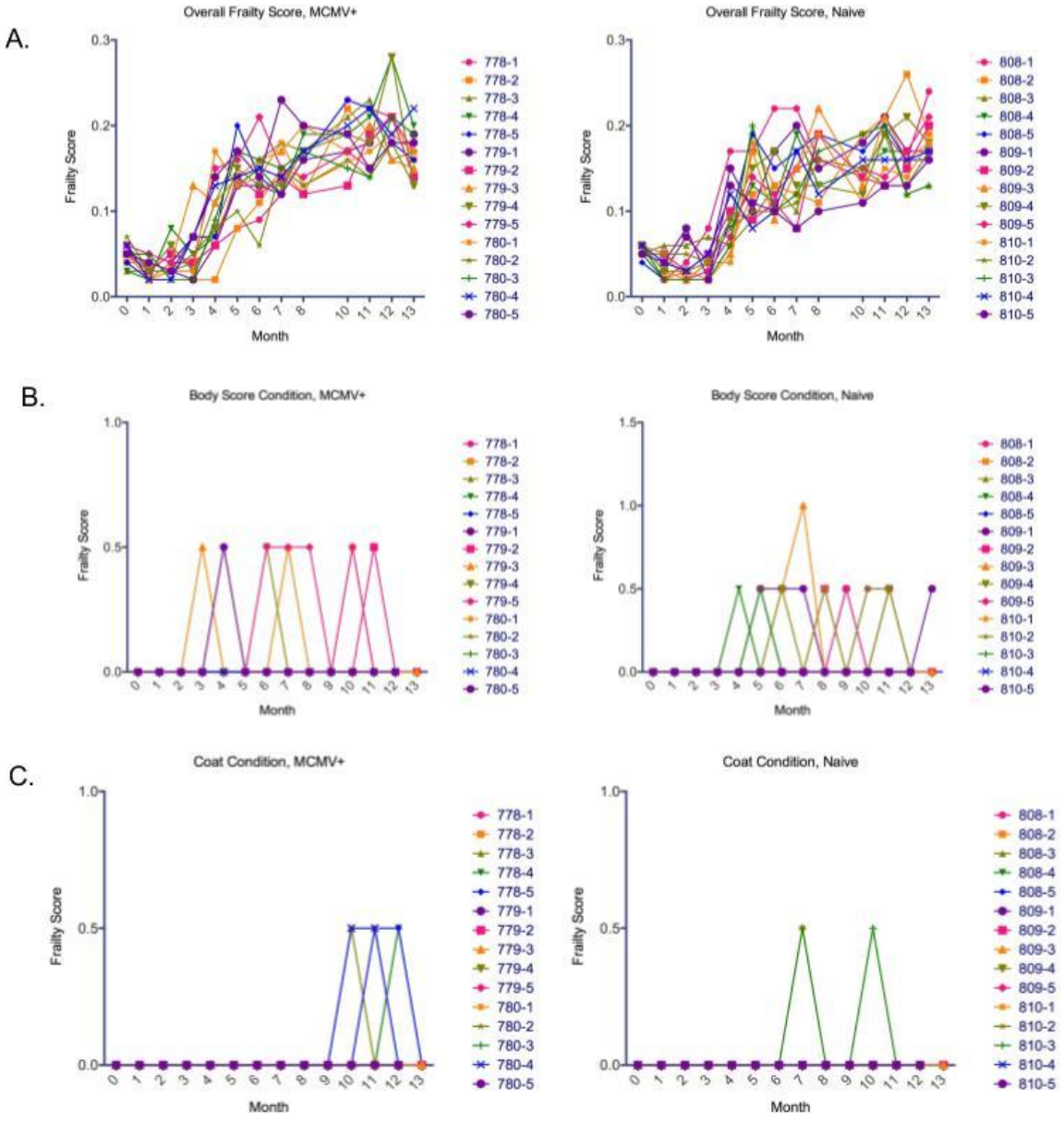
It has been hypothesized that CMV also has a direct effect on frailty syndrome. Murine CMV has been used in mouse models to assess CMV on a short-term, flexible scale. Using the mouse frailty index [13] as a guide, this study explored the effects of CMV and the clinical signs

of frailty in the aging mouse to evaluate whether CMV specifically influences the onset or severity of frailty symptoms in aging mice. These questions are key for better understanding frailty.

Results

Mice were infected at 8 weeks, then followed for 13 months. Peripheral blood was collected each month to stain for T cell subsets as well as antigen-specific cells by flow cytometry. Frailty parameters were assessed monthly using a 23 parameter protocol to monitor the onset and severity of physical symptoms associated with frailty.

Figure 1. *Individual frailty score analysis across all phenotypes*
Each individual mouse was plotted to show the change in frailty score for all parameters in the 13 months following infection. No significant relationships between frailty score and CMV were found.



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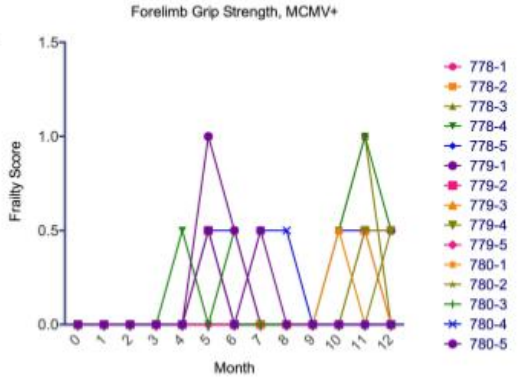
Forelimb Grip Strength, MCMV+
Frailty Score
Month
778-1
778-2
778-3
778-4
778-5
779-1
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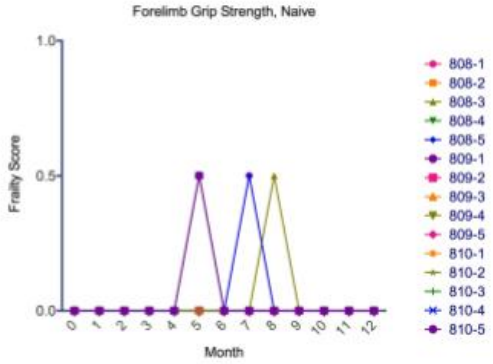
Forelimb Grip Strength, Naive
Frailty Score
Month
808-1
808-2
808-3
808-4
808-5
809-1
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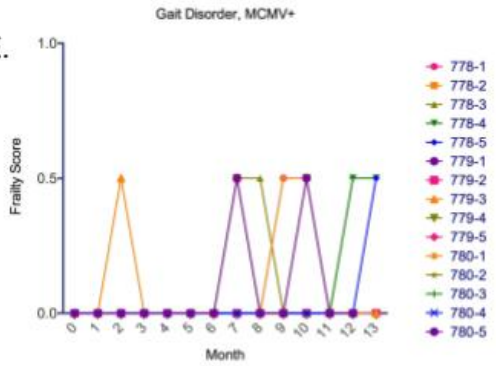
Gait Disorder, MCMV+
Frailty Score
Month
778-1
778-2
778-3
778-4
778-5
779-1
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779-4
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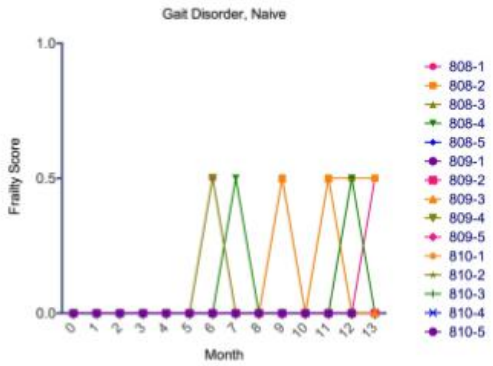
Gait Disorder, Naive
Frailty Score
Month
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808-2
808-3
808-4
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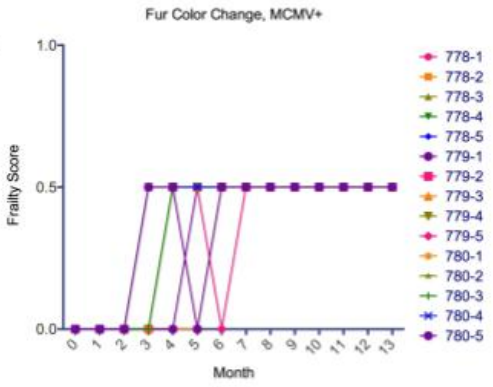
Fur Color Change, MCMV+
Frailty Score
Month
778-1
778-2
778-3
778-4
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780-1
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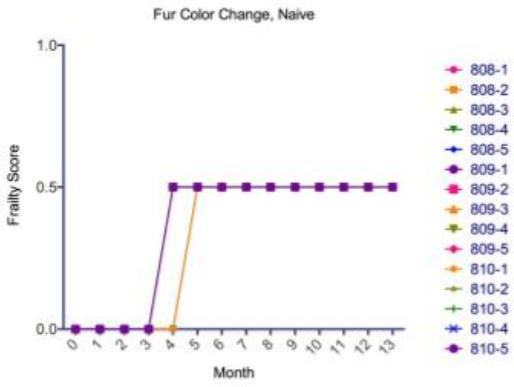
Fur Color Change, Naive
Frailty Score
Month
808-1
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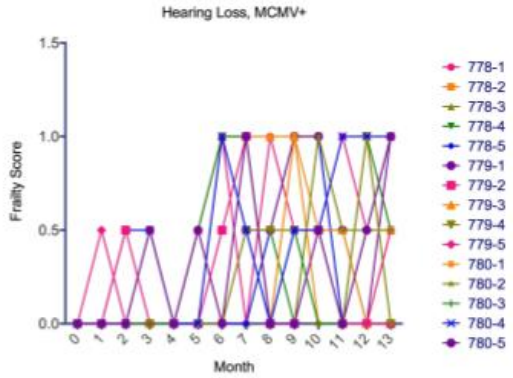
Hearing Loss, MCMV+
Frailty Score
Month
778-1
778-2
778-3
778-4
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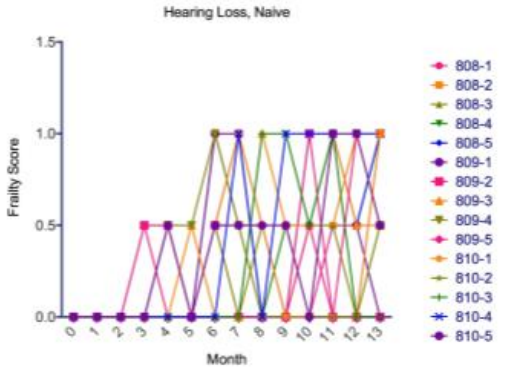
Hearing Loss, Naive
Frailty Score
Month
808-1
808-2
808-3
808-4
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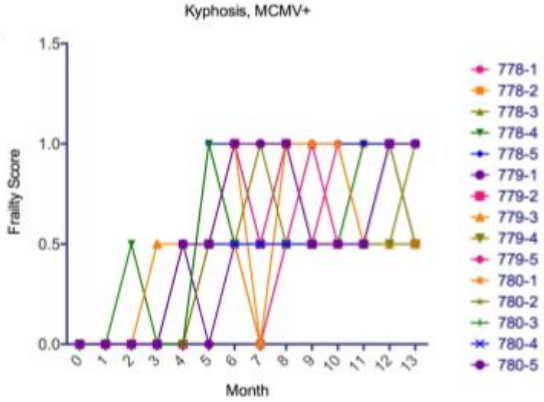
H.


Kyphosis, MCMV+
Frailty Score
Month
778-1
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778-4
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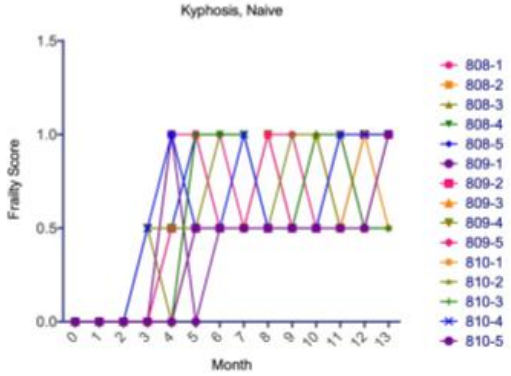
Kyphosis, Naive
Frailty Score
Month
808-1
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808-3
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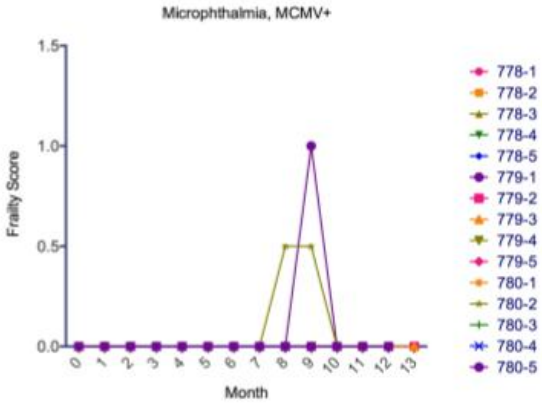
Microphthalmia, MCMV+
Frailty Score
Month
778-1
778-2
778-3
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779-5
780-1
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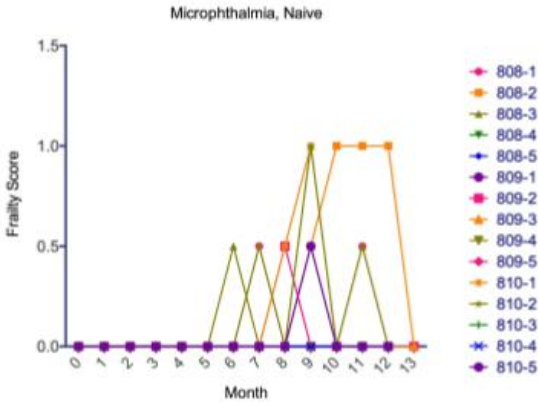
Microphthalmia, Naive
Frailty Score
Month
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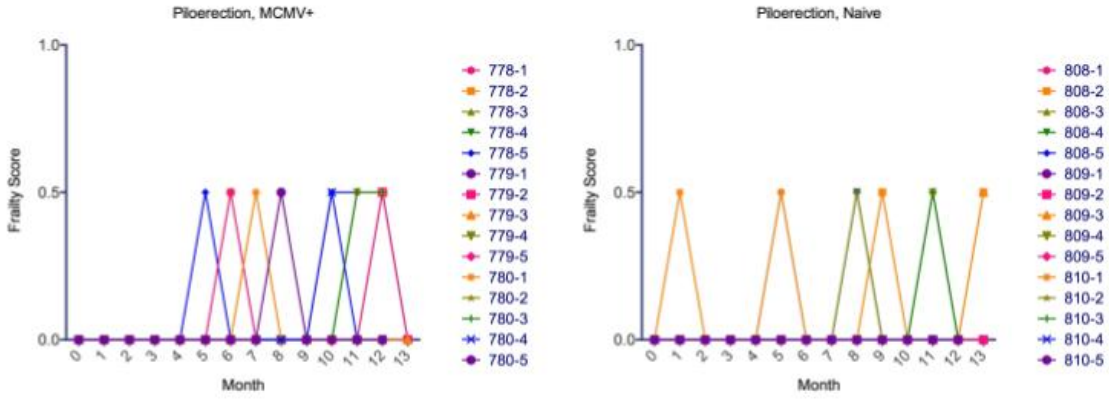
Piloerection, MCMV+
Frailty Score
Month
778-1
778-2
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778-5
779-1
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Piloerection, Naive
Frailty Score
Month
808-1
808-2
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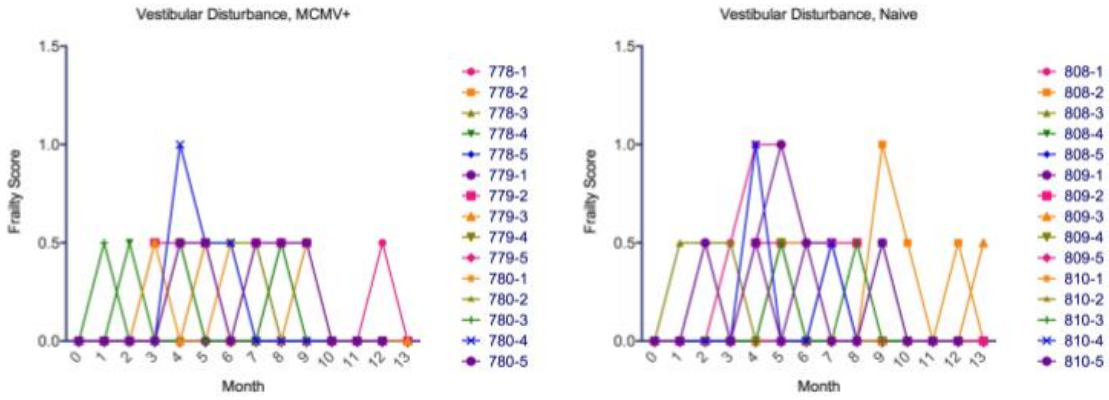
Vestibular Disturbance, MCMV+
Frailty Score
Month
778-1
778-2
778-3
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Vestibular Disturbance, Naive
Frailty Score
Month
808-1
808-2
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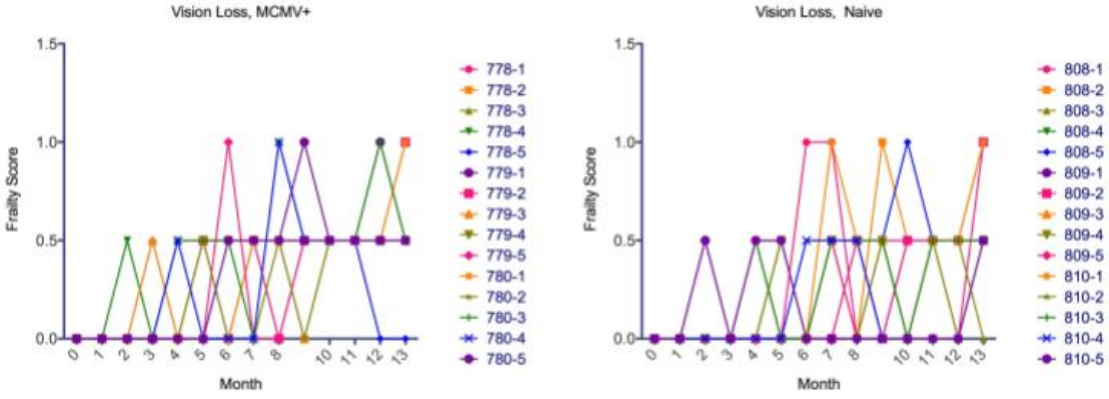
Vision Loss, MCMV+
Frailty Score
Month
778-1
778-2
778-3
778-4
778-5
779-1
779-2
779-3
779-4
779-5
780-1
780-2
780-3
780-4
780-5
Vision Loss, Naive
Frailty Score
Month
808-1
808-2
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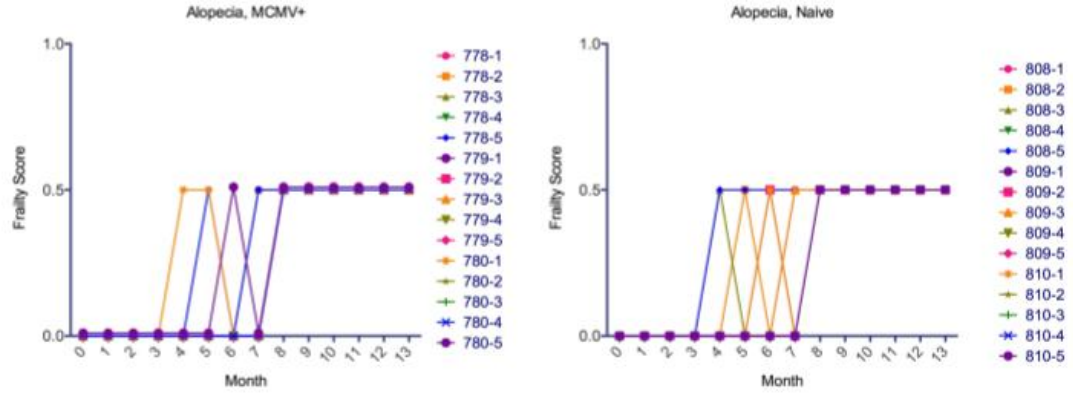
Alopecia, MCMV+
Frailty Score
Month
778-1
778-2
778-3
778-4
778-5
779-1
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779-4
779-5
780-1
780-2
780-3
780-4
780-5
Alopecia, Naive
Frailty Score
Month
808-1
808-2
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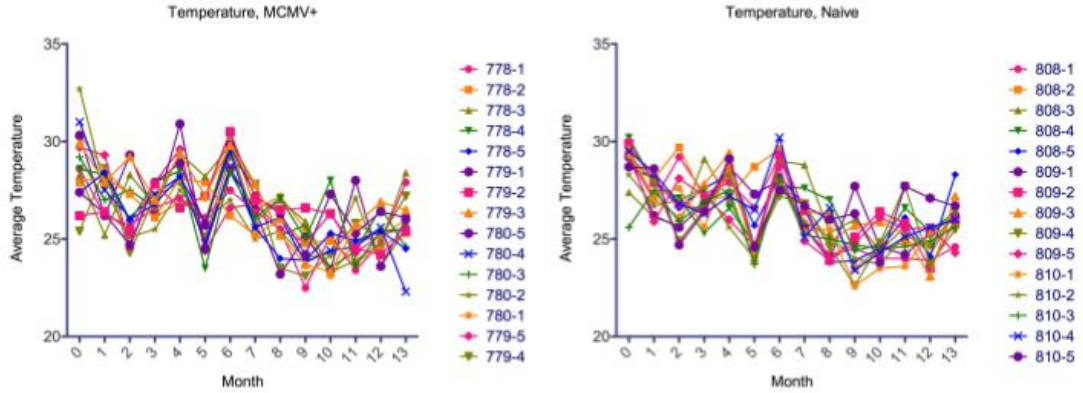
Temperature, MCMV+
Average Temperature
Month
778-1
778-2
778-3
778-4
778-5
779-1
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780-3
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779-4
Temperature, Naive
Average Temperature
Month
808-1
808-2
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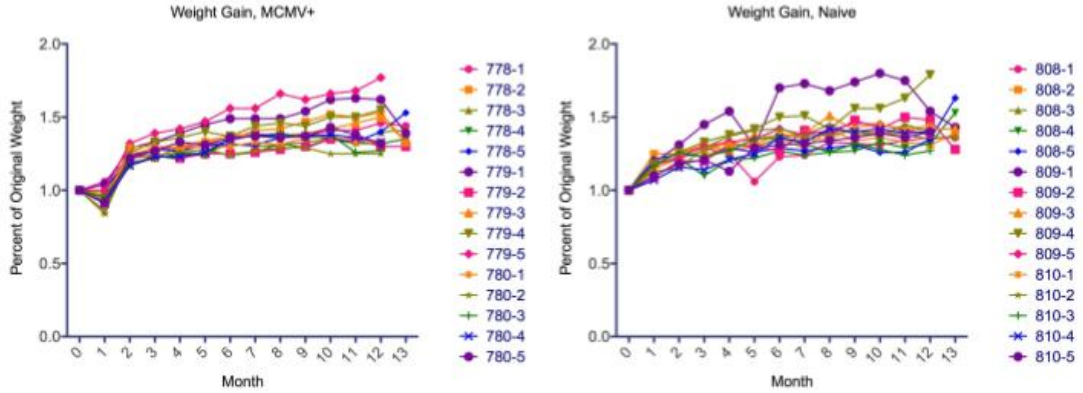
Weight Gain, MCMV+
Percent of Original Weight
Month
778-1
778-2
778-3
778-4
778-5
779-1
779-2
779-3
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779-5
780-1
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Weight Gain, Naive
Percent of Original Weight
Month
808-1
808-2
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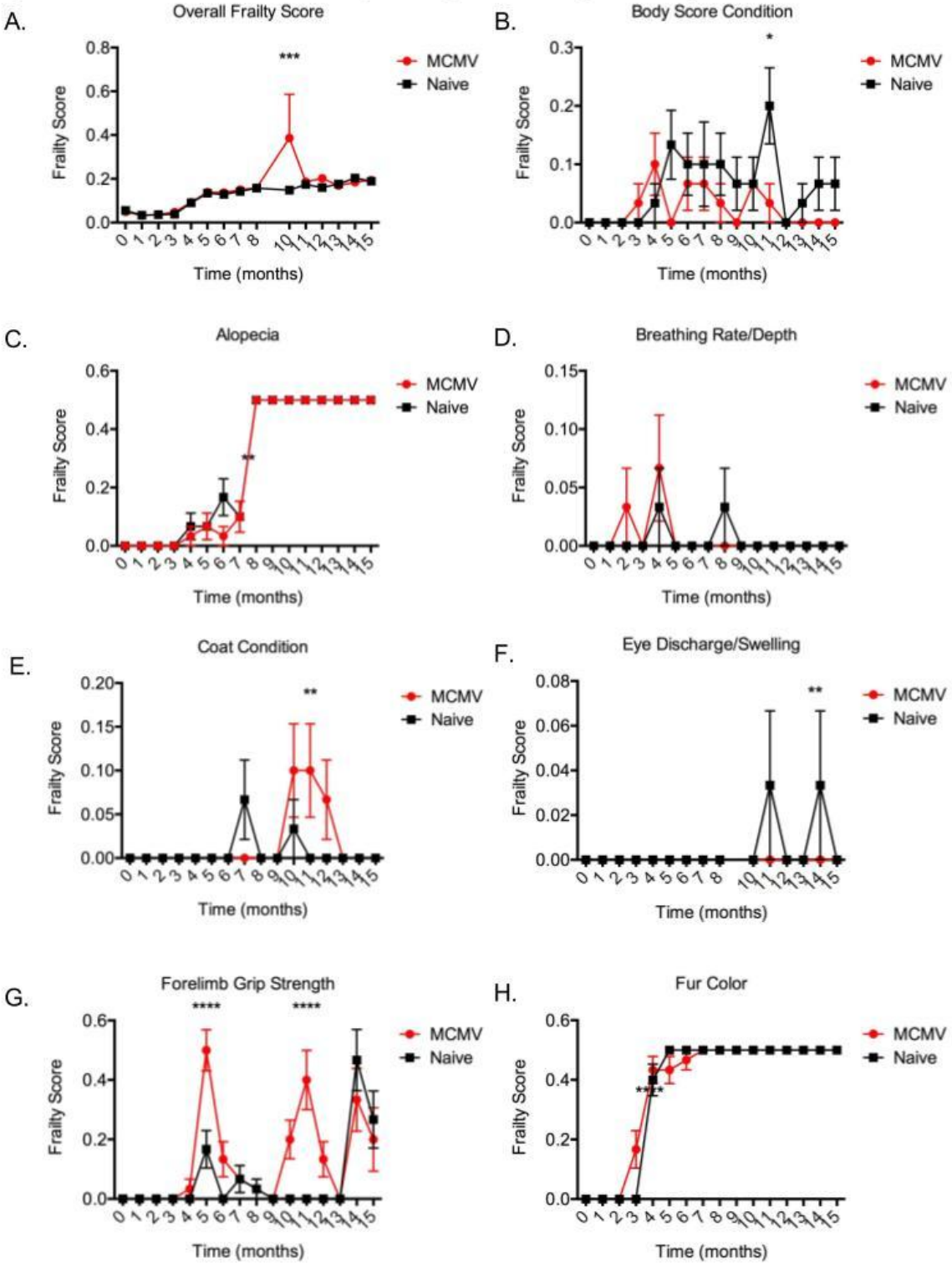
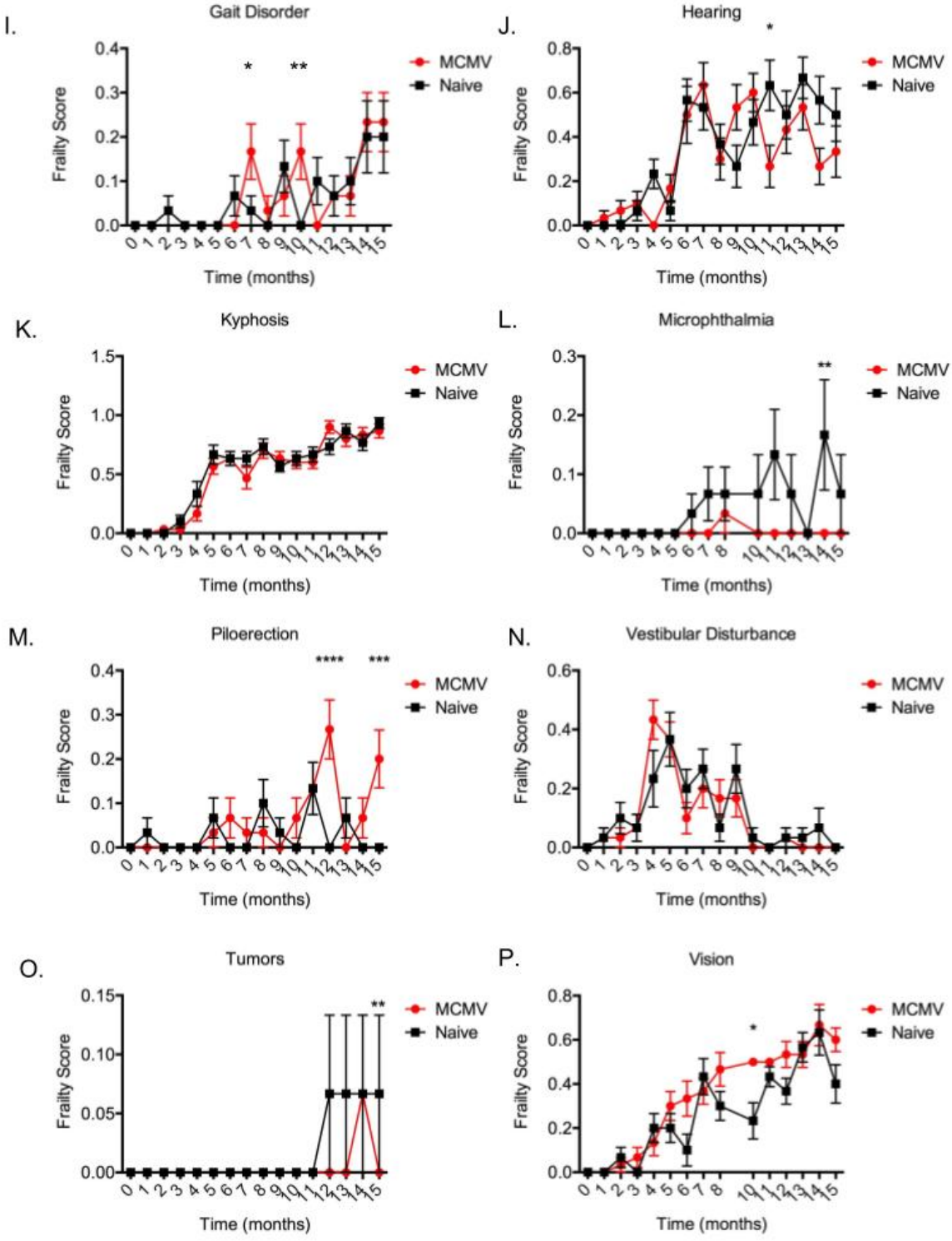
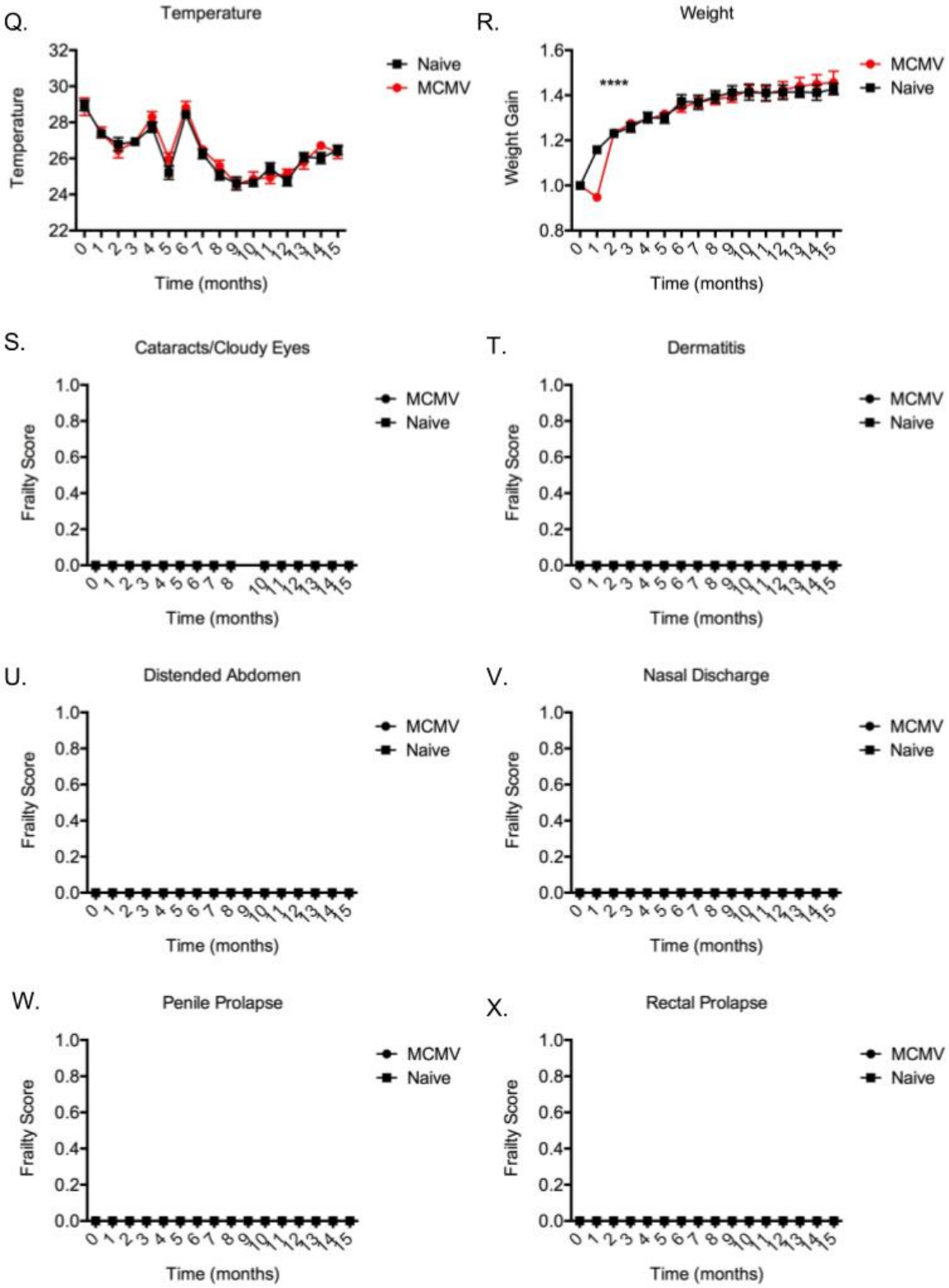
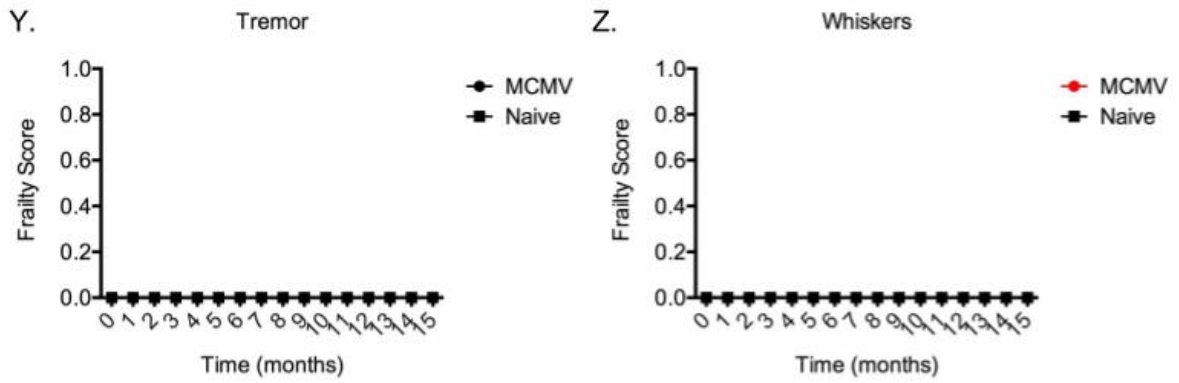
Figure 2. *Frailty index of MCMV+ mice and naive mice*
The combined groups of mice, MCMV+ (n=15) and naive (n=15), were plotted to show the changes in frailty score for all parameters against each other over the full 15 months of investigation. No significant relationship between frailty and CMV were found.




I.
Gait Disorder
Frailty Score
Time (months)
MCMV
Naive
J.
Hearing
K.
Kyphosis
L.
Microphthalmia
M.
Piloerection
N.
Vestibular Disturbance
O.
Tumors
P.
Vision


Q.
Temperature
Temperature
Time (months)
Naive
MCMV
R.
Weight
Weight Gain
****
Time (months)
MCMV
Naive
S.
Cataracts/Cloudy Eyes
Frailty Score
Time (months)
MCMV
Naive
T.
Dermatitis
Frailty Score
Time (months)
MCMV
Naive
U.
Distended Abdomen
Frailty Score
Time (months)
MCMV
Naive
V.
Nasal Discharge
Frailty Score
Time (months)
MCMV
Naive
W.
Penile Prolapse
Frailty Score
Time (months)
MCMV
Naive
X.
Rectal Prolapse
Frailty Score
Time (months)
MCMV
Naive



Overall frailty scores increased steadily between both MCMV and naive mice populations (Fig 1 & 2). A significant increase in frailty in MCMV+ mice was seen at 10 months, which fell back in line with initial progression at month 11. For most frailty phenotypes, both infected and uninfected mice increased in frailty at matching, steady rates. In some phenotypes (coat condition, fur color change, gait disorder, forelimb grip strength, piloerection, vision loss) MCMV infected mice showed increased levels of frailty over naive populations. However, in some categories, naive mice showed more frailty symptoms than the MCMV mice. In many of the categories, there were no results for either population of mice to report. In general, the phenotypes that the mice have no control over (such as fur loss and fur color changes) exhibited identical patterns of increasing frailty as the disease progressed. In categories that the mice did have some control over (coat condition, forelimb grip strength, etc.) there were distinct differences between populations at periods during infection. Previous studies in humans have found that frailty syndrome has some distinct symptoms, such as unintentional weight loss, weakness, and slow walking speed [7, 17]. Latent viral infection, like CMV, has also been linked to cognitive decline over time [18]. In the MCMV infected mice, the symptoms associated with some of these changes (gait disorder, weight, and forelimb grip strength) did show increased

levels of frailty (or decreased, in the example of weight gain; where the MCMV infected mice showed a drop in weight following infection, and then normalized weight gain in conjunction with naive mice) in the duration of the infection. This quicker progression of symptoms contributed to the increased overall frailty score spike at ten months for the MCMV infected mice, but evened out over the course of the study as other frailty factors began to affect all mice equally, raising the overall frailty score of all the mice in both populations.

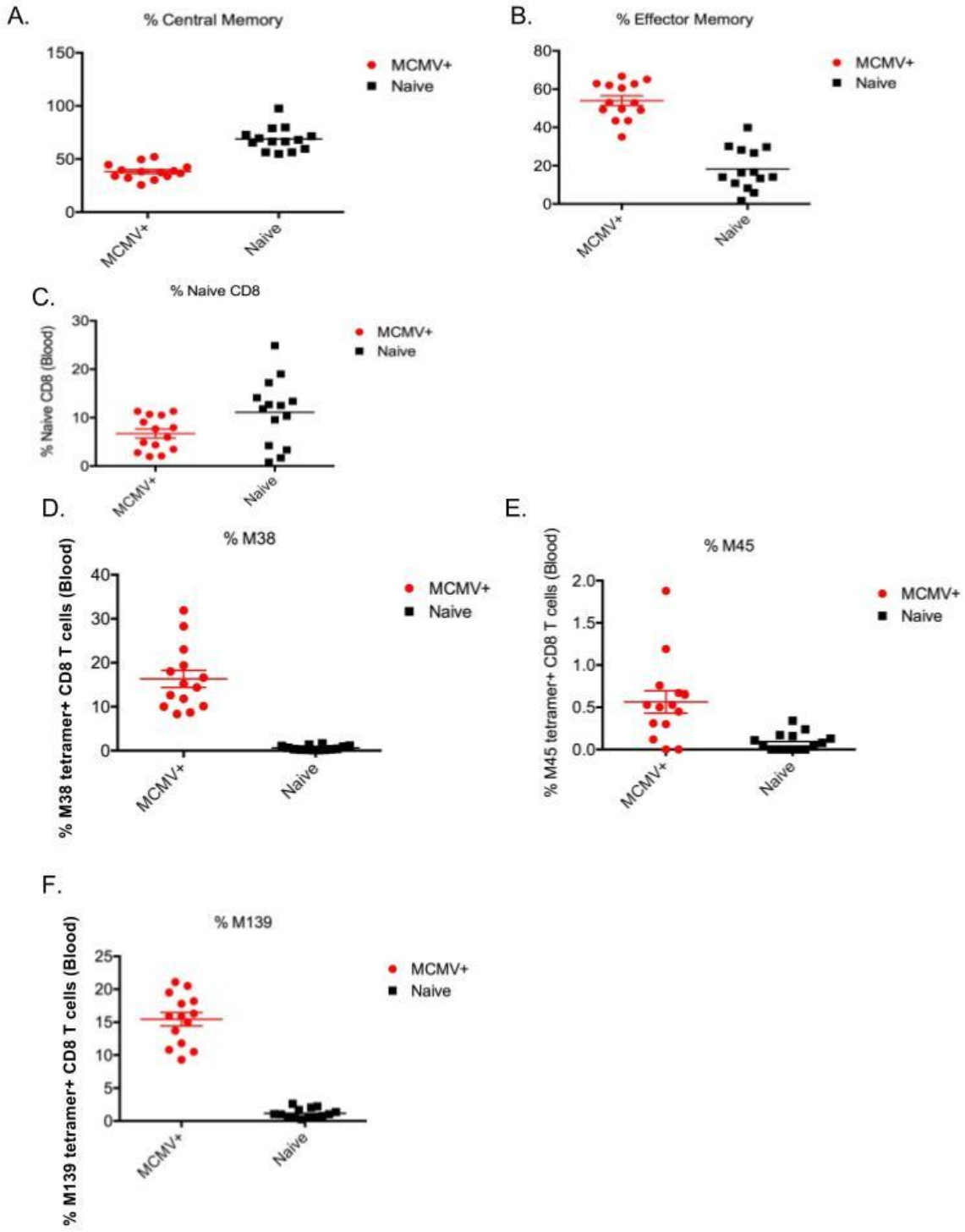


Figure 3. *T Cell and tetramer levels following MCMV infection after 9 weeks*. (A) Decreased level of central memory T cells in MCMV+ mice (B) Increased levels of effector cell poopulations in MCMV+ mice (C) Decreased levels of naive T cells in MCMV+ mice (E) M38 tetramer levels elevated in MCMV+ mice (E) Slight increase (0.5% of CD8 T cells) in M45 tetramer in MCMV+ mice (F) Elevated m139 tetramer levels in MCMV+ mice

Mice infected with MCMV+ showed increased levels of effector memory T cells following infection, while simultaneously showing decreased levels of central memory T cells (Fig 3). These findings are consistent with previous studies [10, 14, 15] that show initial increase of M38, M45, and m139 tetramers following infection. In B6 mice, populations of M38 and m139 inflate dramatically over the first few weeks post infection [8, 15]. In contrast, M45 levels form stable memory T cell populations that express a central memory T cell phenotype.

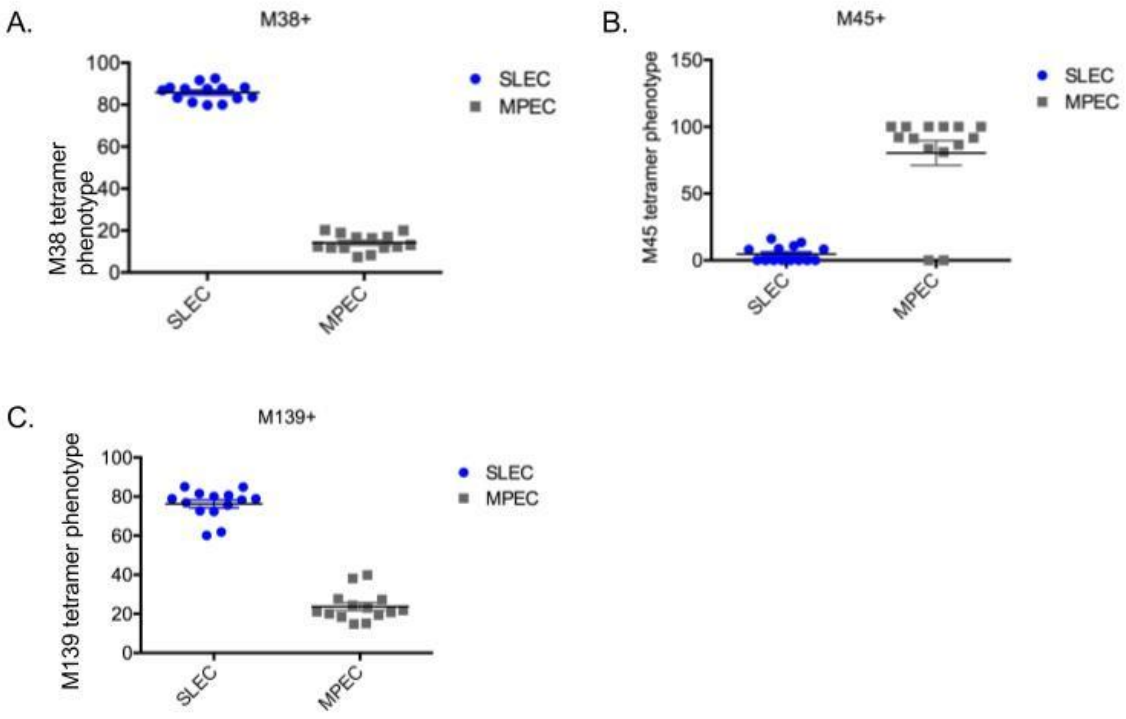


Figure 4. *SLEC/MPEC ratio of key viral proteins studied within the CMV subset.* (A) M38+ epitope contains elevated SLEC levels over MPEC (B) M45+ epitope shows higher levels of MPEC over SLEC (C) m139+ epitope shows increased SLEC levels over MPEC

In B6 mice, the inflammatory M38 and m139 have elevated frequencies of short-lived effector cells (SLECs) that have high effector function in controlling the virus, while have lower levels of memory precursor effector cells (MPECs) that are intended to form memory cells [10]. While M45 is typically immunodominant at the peak of acute response, at chronic infection it is noninflammatory in B6 mice [14, 16]. M45 levels in these mice will show increased levels of

MPECs over SLECs during the duration of infection and appear to be more similar to classic central memory T cells [8], as shown in Figure 4.

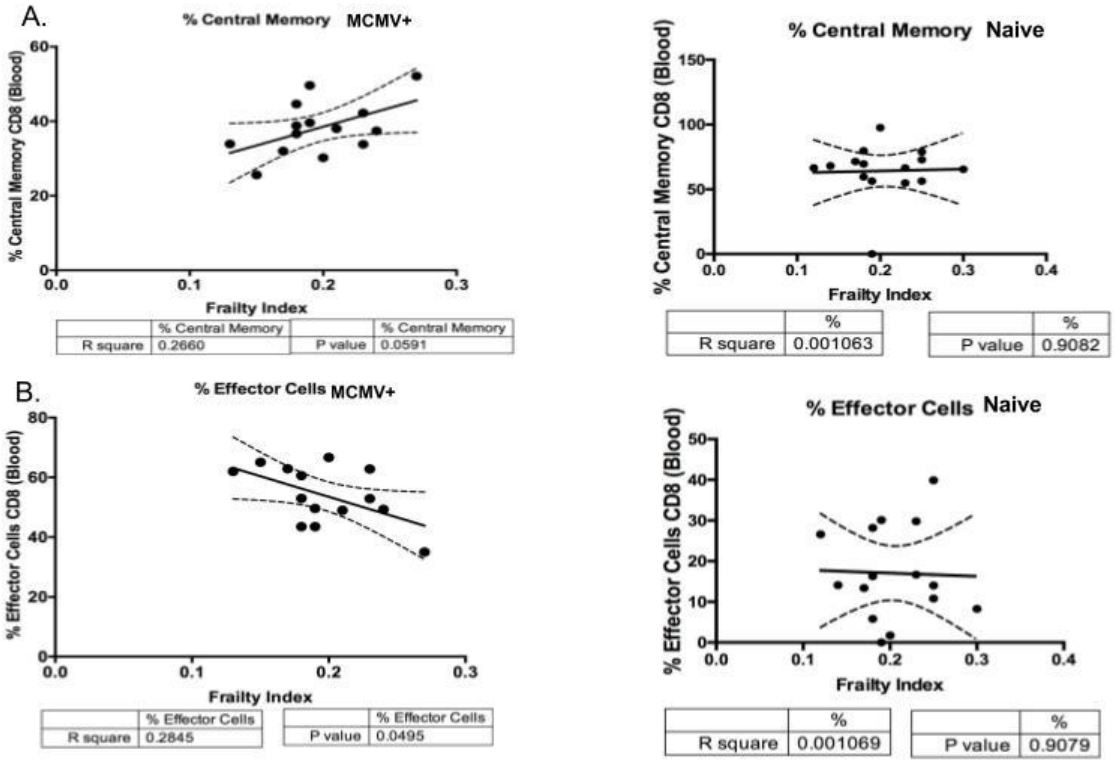


Figure 5. *Changes in levels of central and effector memory T cells as a result of MCMV infection and frailty, 13 months post-infection.* (A) Increased central memory cell population in each individual infected mouse with increasing frailty. (B) Decreased effector cell population in infected mice with increasing frailty
Left panels shows individual MCMV+ infected mice. Right panels shows individual naive mice.

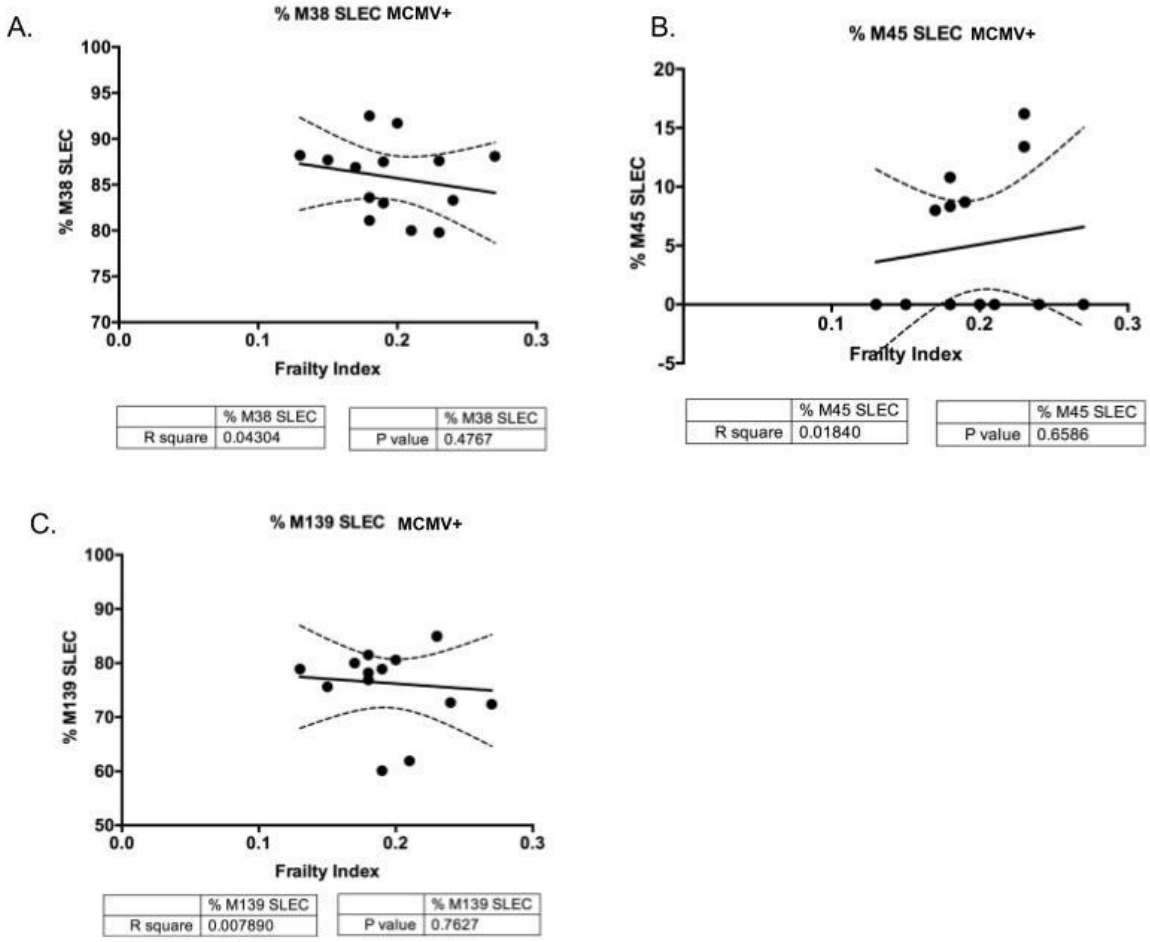


Figure 6. *Tetramer SLEC levels with frailty index.* (A-C) Each individual infected mouse was plotted for their CMV specific tetramer levels against their frailty score. Overall, no relationship was found between the frailty score and the frequency of CMV specific T cells.

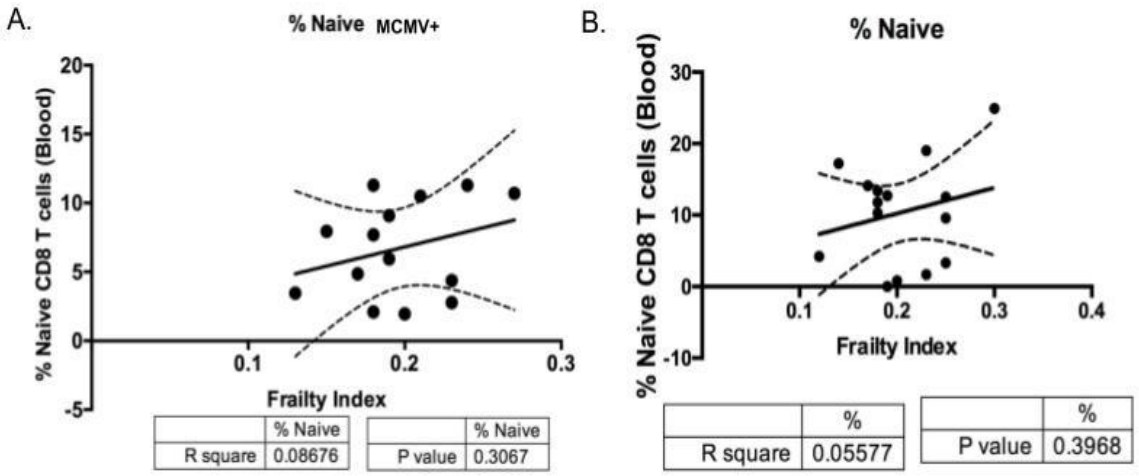


Figure 7. *Naive and Tetramer population against frailty.* (A-B) The percent of naive T cells in both MCMV infected and naive mice remained low as frailty for the infected mice increased, but overall increased with increasing frailty

Overall assessment was done on whether the magnitude of the immune response to CMV was related to the frailty index score of the mice over 13 months post-infection (Figs 5, 6 & 7). In relation to frailty, there were no major responses in any categories of immune response to CMV that would indicate a relationship to frailty. Overall, central memory T cells remained lower in MCMV+ mice and effector cell population remained higher at all levels of frailty (Fig 5). Additionally, M38 and m139 tetramer populations remained high in infected mice, while M45 tetramer populations remained low and hardly fluctuated with changes to frailty (Fig 6). Even with changes in frailty, the overall naive and tetramers populations in the infected mice did not fluctuated, but instead remained steady (Fig 7).

Discussion:

The mice in the study exhibited the same biological markers as previous studies have found in murine CMV (MCMV): high SLEC levels for M38 and m139, and increased effector and central memory T cells over time. The central and effector memory cell populations

remained inflated for MCMV+ mice even with increased frailty, but steadily decreased as frailty increased. In individual mice, central memory cells went up with increased frailty while effector memory cells decreased with increased frailty as seen with persistent infection. However, there was no relationship found between the frailty score and the frequency of CMV specific T cells.

Further, CMV did not make an impact on the severity or length of time to acquire frailty. Mice in both uninfected and infected populations acquired frailty symptoms at a steady and similar rate. Around month ten there were some parameters (gait disorder, piloerection, forelimb grip strength) and the overall frailty score showing that the MCMV+ mice were spiking in their frailty, possibly deteriorating faster than the uninfected mice. However they recovered in later months after possibly adjusting to their disease once more, following the frail course of the uninfected mice.

The lack of detectable, clinical signs of CMV seems to extend further to the clinical signs of frailty. Physicians will not be able to assess a patient based on frailty and automatically correlate their frailty index to CMV. Additionally, physicians will not be able to gauge how well/poorly a patient with long term CMV is doing based on the traditional signs of aging and frailty. The increased inflammatory response to the infection still poses a serious concern to the medical field, and its rate of prevalence in adults calls for research into finding a vaccine to prevent congenital infections and age-related diseases. While CMV may not have a direct link to the clinical signs of frailty, the biological effects and long-term illnesses that result from the weakened immune system continues to call for action against this virus.

Methods:

Mice

Male C57Bl/6J mice from Jackson Labs (stock # 000664) were utilized in this study.

Infection

At 8 weeks of age, n=15 mice were infected with $1x10^5$ pfu MCMV (Smith strain) in 100 ul of PBS, by intraperitoneal injection. Age matched controls were left uninfected.

Flow cytometry

To evaluate the T cell subsets and the MCMV-specific CD8 T-cell response, peripheral blood lymphocytes were stained with fluorochrome-conjugated antibodies specific for CD4 (GK1.5), CD8 (53-6.7), CD44 (IM7), CD62L (MEL-14), and KLRG1 (2F1), as well as $m139_{419-426}$:K^b, $M45_{985-993}$:D^b, and $M38_{316-323}$:K^b tetramers (National Institutes of Health Tetramer Core Facility, Emory University, Atlanta, GA), then evaluated by flow cytometry. Data acquisition was performed on a custom-made, four-laser BD Fortessa flow cytometer (Becton Dickinson, Sunnyvale, CA), and was analyzed using FlowJo software (Tree Star, Inc., Ashland, OR). A minimum of 10,000 $CD8^+$ events within the lymphocyte gate was collected for all files.

Clinical Frailty Assessment:

Each subset of mice was subjected to monthly clinical frailty assessments following Table 1 for 15 months. Assessments increased during the last month of the trial. The table was modified following a previous study [13] analyzing and compiling details of clinical frailty in aging B6 mice. Scores were given for each of the 23 factors, 0 showing no frailty symptoms, 0.5 showing mild effects, and 1.0 showing severe effects. Details of the evaluation of these parameters (with the exception of weight and temperature, outlined in Table 3) and how to score are outlined in Table 2. Temperature and weight scores were determined using standard

deviations from a typical B6 adult mouse temperature and weight. If a mouse fell within 1 standard deviation, it was given a score of 0.25. If it fell between 2 standard deviations, it was given a score of 0.5. If it fell between 3 standard deviations, it was given a score of 0.75. If it fell outside of 3 standard deviations, it was given a full score of 1.0.

Most clinical signs of frailty can be found visually by observing the animal for the deficits. Exceptions are as follows: Temperature was taking with an industrial precision laser thermometer pointed at the abdomen, taking the average of three measurements. Weight was measured using a standard gram scale. Kyphosis was determined by observing overall hunchbacked posture that may affect gait, and feeling for exaggerated curvature along the spine. Hearing loss was taken using an animal training clicker to administer three clicks a few seconds apart. Vision loss was determined by raising and bringing a mouse closer to the ground by the tail. The correct response is to reach for the ground at all heights. Vision failure starts when the mouse fails to reach for the ground, even as it approaches the grate. Forelimb grip strength was measured by allowing the mouse to grip onto a grate, and then attempting to pull it off. The strength and whether or not the mouse stays gripped indicated the level of deterioration of grip strength. Further details about the scoring can be found in Table 2.

Table 1: Clinical signs of frailty in aging B6 mice

Clinical Symptom	Description of symptom deficit
Alopecia	Fur loss due to age-related balding and/or barbering
Fur color change	Change in fur color from black to gray/brown
Dermatitis	Inflammation, over-grooming, barbering or scratching that results in skin erosion. Open sores anywhere on body

Loss of whiskers	Loss of whiskers due to aging and/or whisker trimming
Coat Condition	Ruffled and/or matted fur. Ungroomed fur. Coat does not look smooth, sleek, and shiny
Piloerection	Involuntary bristling of the fur due to sympathetic nervous system activation
Cataracts/Cloudy eyes	Clouding of the lens of the eye; opaque spot located in the center of the lens
Eye Discharge/Swelling	Eyes are swollen or bulging; abnormal secretions and/or crusting
Microphthalmia	Eyes are small and/or sunken in one or both eyes
Nasal Discharge	Abnormal discharge from the nasal passages
Breathing Rate/Depth	Difficulty breathing, pulmonary congestion, and/or rapid breathing when undisturbed
Kyphosis	Exaggerated outward curvature of the lower cervical/thoracic spine (neck to back region); increased hunchback posture
Gait Disorder	Lack of coordination in movement which include: hopping, wobbling, uncoordinated gait; wider stance; circling or weakness
Tremor	Involuntary shaking at rest or during movement
Body Score Condition	Visual signs of muscle wasting or severe obesity based on amount of flesh covering the bones
Hearing Loss	Failure to respond to sudden sound
Tumors	Development of tumors or masses anywhere on the body
Distended Abdomen	Enlarged abdomen. May be due to a tumor, organ enlargement, or fluid accumulation
Rectal Prolapse	Protrusion of the rectum just below the tail
Penile Prolapse	Protrusion of the penis so that it cannot reenter the penile sheath
Forelimb Grip Strength	A decline in the strength of forelimb grip
Vestibular disturbance	Disruption in the ability to perceive motion and gravity due to malfunction of the vestibular system; reflected in problems with balance, orientation, and acceleration

Vision Loss	Loss of vision indicated by failure to reach out for the ground when lowered by the tail
Weight (g)	Increase or decrease in overall body weight
Temperature (°C)	Increase or decrease in overall body temperature

Table 2: Evaluation and scoring methods for B6 mouse

Clinical Symptom	No deficit (0)	Mild deficit (0.5)	Severe deficit (1.0)
Alopecia	No fur loss	Fur loss on ≤25% of body	Fur loss on >25% of body
Fur color change	No color change	Brown/gray fur on ≤25% of body	Brown/gray fur on >25% of body
Dermatitis	No dermatitis	Dermatitis on ≤25% of body	Dermatitis on >25% of body
Loss of whiskers	Full set of whiskers	Thinning whiskers	Complete loss/Very few whiskers
Coat Condition	Clean, shiny, groomed	Slightly unkempt, slight grooming attempt	Completely disheveled, no attempt at grooming
Piloerection	No piloerection	Slight piloerection on the mouse, isolated to small patch/area	Complete piloerection of body
Cataracts/Cloudy eyes	Clear eyes	Small patch of clouding/spots on one eye	Clouding on both eyes and/or complete clouding of one eye
Eye Discharge/Swelling	No swelling/discharge	Slight swelling/discharge around one eye	Severe swelling/discharge on one or both eyes
Microphthalmia	Eyes normal	One eye small or slightly sunken	One eye completely shut and/or both eyes sunken
Nasal Discharge	Nose normal	Slight nasal discharge	Severe nasal discharge
Breathing	Breathing rate normal	Intensified breathing rate	Intensified breathing

Rate/Depth		at rest	rate at rest that worsens with movement
Kyphosis	No curvature of the spine	Slight curvature of the spine, could be unseen to naked eye	Extreme curvature of spine and visible hunchback appearance
Gait Disorder	Normal gait	Slight wobble/hoppy gait	Severe wobble/hoppy gait
Tremor	No tremor	Slight tremor that may come in spurts	Severe tremor; constant
Body Score Condition	Proportioned muscle and fat distribution	Slightly disproportionate muscle/fat distribution	Severely disproportionate muscle/fat distribution
Hearing Loss	Response to all 3 clicks	Response to 1-2 clicks	No response
Tumors	No tumors	Small, single tumor	Multiple tumors, enlarged tumor
Distended Abdomen	No swelling of the abdomen	Slight swelling	Severe swelling
Rectal Prolapse	No prolapse	Slight prolapse	Severe and inflamed prolapse
Penile Prolapse	No prolapse	Slight prolapse	Severe and inflamed prolapse
Forelimb Grip Strength	Complete grip on grate, does not let go	Grip maintained for short period of time, becomes weak and loses grip	No grip at all, pulled off easily
Vestibular disturbance	Normal balance and orientation	Slight disturbance in ability to balance and orient itself	Severe balance and coordination problems, no clear orientation
Vision Loss	Reaches for ground immediately at all heights	Only reaches when close (~1 inch away or less) from the ground	Does not reach for ground, nose will hit before it reaches at all

Table 3: Weight and temperature scoring guideline for B6 mice

	0.25 score	**0.5 score**	**0.75 score**	**1.0 score**
Weight (g)	$25.8 \leq x \leq 28.8$	$24.3 \leq x \leq 30.3$	$22.8 \leq x \leq 31.8$	Outside 0.75 parameters
Temperature (°C)	$25.9 \leq x \leq 28.9$	$24.4 \leq x \leq 30.4$	$22.9 \leq x \leq 31.9$	Outside 0.75 parameters

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