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Validation of Metabolic and Immunologic Biomarkers TNF-a, IGF, IL-6, CRP and Hair Cortisol in the Common Marmoset

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Validation of Metabolic and Immunologic Biomarkers *TNF-a, IGF, IL-6, CRP* and Hair *Cortisol* in the Common Marmoset

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Abstract

The common marmoset is a good model for research because they are easy to house and have complex social relationships (French et al., 2019). Marmosets are sensitive to social isolation, and when introduced to a stressor, the HPA axis is activated (Saltzman & Abbott, 2011). The purpose of this experiment is to validate marmosets as a translational model for stress due to social relationships in humans. This is done by validating biomarker concentration levels at baseline, then comparing the concentration when introduced to a stressor. The biomarkers *IL-6, CRP, IGF-1* and *TNF-a* were tested using a serum assay, then running the well plate under a plate reader to determine the concentration. No antibodies were detected in the marmoset samples for the biomarkers *IL-6, CRP, IGF-1* and *TNF-a.* Chronic *cortisol* concentration was determined using marmoset hair samples. The high stress group for hair *cortisol* concentration was significantly greater than the control group, providing evidence that hair cortisol is a reliable biomarker for long-term chronic stress. Marmosets can be used as a translational animal model to further understand and study both acute and chronic stress due to social relationships and isolation.

Keywords*: marmoset, biomarker, stress, social isolation, serum assay, hair cortisol concentration*

Validation of Metabolic and Immunologic Biomarkers *TNF-a, IGF, IL-6, CRP* and Hair *Cortisol* in Common Marmosets

The common marmoset (*Callithrix jacchus*) is a small New World primate native to coastal forests in South America (Kishi et al., 2014). Marmosets provide an excellent model for biomedical research as they are relatively small (300-500 g), thus easy to house in laboratory settings, as well as having strongly developed information on their brain anatomy and functions (French et al., 2019). Varying from other nonhuman primates, marmosets live in extended family units consisting of a monogamous breeding pair, their offspring, and adult relatives, all of which take responsibility in child-rearing (French et al., 2008).

In stressful conditions, the hypothalamic-pituitary-adrenal (HPA) axis promotes survival by focusing on essential functions, such as metabolism, immunity, and cardiovascular activity, while down-regulating non-essential functions such as reproduction and parental behavior (Saltzman & Abbott, 2011). The hypothalamus releases *corticotrophin-releasing hormone (CRH)*, which in turn causes the adrenal gland to increase the production of glucocorticoids. Marmosets are sensitive to social separation and isolation, which creates a strong "stress response" via activation of the HPA axis. Chronic, prolonged stress can have adverse effects by negatively affecting autonomic, neuroendocrine, metabolic, and immune systems (Lupien et al., 2009).

Interleukin-6 (IL-6) is a pleiotropic cytokine biomarker that regulates immune responses, inflammation, and hematopoiesis (Kimura & Kishimoto, 2010). Along with stimulatory effects on hepatocytes, *IL-6* exhibits inhibitory effects on antiviral antibody response. *C-reactive protein* (*CRP*) is an inflammatory biomarker that has been proposed to predict future cardiovascular risk by evaluating plasma levels of *CRP* of healthy men and women (Albert et al., 2001). *Insulin-like*

growth factor-I (IGF-1) is a polypeptide biomarker that is structurally similar to insulin and promotes cell replication (Bhagavan, 2002). Components of *IGF-1* can be suppressed with pro-inflammatory cytokines such as *tumor necrosis factor-α (TNF-α*) (Hellgren et al., 2018).

TNF-α is an inflammatory cytokine biomarker associated with inflammatory-mediated angiogenesis, or development of new blood vessels (Hellgren et al., 2018). It is produced by macrophages, monocytes, neutrophils, activated lymphocytes, astrocytes, and many other cells and is unregulated in cases of human obesity and insulin resistance (Jameson, 2016). Marmosets experience increased *cortisol* levels, one of the main glucocorticoids in many mammals, when associated with stressors such as human activity, relocation, or separation from their partner or family unit (Ash et al., 2017). Hair is useful for determining chronic *cortisol* concentration in the past several months, whereas blood *cortisol* concentration reflects acute *cortisol* concentration produced in the last several minutes and urine reflects *cortisol* produced in the last couple hours.

Biomarkers *IL-6, CRP*, *IGF-1*, *TNF-α*, and *cortisol* are present in humans, but it is not known if they are also present in the common marmoset. In this study, we examine if marmosets, like humans, express biomarkers *IL-6, CRP, IFG-1, TNF-α* and *cortisol* in order to develop a methodology to measure the level of those biomarkers by utilizing blood and hair assays. Another factor we examined is if those biomarkers are present, will the level present change by either increasing or decreasing in comparison to the baseline after the marmoset is exposed to a stressful situation such as isolation.

An additional purpose of this study is to validate marmosets as a translational model for neuroscience research regarding psychogenic stress due to social relationships. Marmosets are an excellent animal model due to their complex central nervous system and because primates have homologous genetics, development, and neural architecture to humans (French et al., 2008). If

biomarkers *IL-6, CRP, IFG-1*, *TNF-α* and *cortisol* are present in marmosets, then the concentration will increase when introduced to a stressor compared to the baseline. The marmoset model can be further developed for clinical research regarding how stress affects cell reproduction, angiogenesis, antibody response, and future cardiovascular risk.

Methods

Subjects. Fifteen (eight female and seven male) white-tufted ear marmoset (*Callithrix jacchus*) housed at University of Nebraska at Omaha in the Callitrichid Research Center served as the animal subjects of this experiment. The marmosets were divided into three groups based on stressors experienced in previous experiments during summer 2019. The "high stress" group (n = 5) were part of a long-term social stress project that led to chronic stress due to isolation. The "low stress" group (n = 5) were part of a partner preference project that resulted in minimal stress. The control group (n = 5) were not involved in any recent stress-causing projects.

Blood Serum Assay. Blood was collected from marmosets during veterinary checks, spun down, and the serum frozen until needed. Assays for biomarkers *IL-6, CRP, IGF-1* and *TNF-α* were performed according to the instructions included in the pre-made assay well plates. The well plates were then run under the plate reader to determine the concentration of the respective biomarker compared to the standard.

Hair Cortisol Concentration. Hair was collected from marmosets by shaving 1-2 inches at the base of the tail using a motorized razor. The hair sample was weighed using a scintillation vial with cap, then prepared by washing in 70% isopropyl alcohol for three minutes, pouring out the excess, and repeating once. After letting the sample air dry, 2 mL of methanol was added to the vial. Using surgical scissors, the hair was cut in the scintillation vial into very small and uniform pieces. With the caps on, the vials were placed on a shaker for approximately 12-15 hours. Once

the methanol evaporated in a ventilated hood, the sample was resuspended in 200 mcl of PBS. The samples were vortexed, then an ELISA assay was performed with diluted concentration samples.

Results

The blood serum assays for biomarkers *IL-6, CRP, IGF-1* and *TNF-α* did not detect any antibodies in the marmoset samples compared to the standards. For the hair *cortisol* concentration, marmosets in the social stress (high stress) experiment displayed a significantly higher level (*$H(3,12) = 7.27$, $p < 0.05$) of hair *cortisol* compared to marmosets who participated in the partner preference (low stress) experiment and compared to the control group according to the Kruskal-Wallis test (Figure 1). The partner preference and control group did not have a significant difference. The hair *cortisol* concentration did not have a significant difference (*$U = 21$, $p > 0.05$) between males and females subjects according to the Mann-Whitney U test (Figure 2).

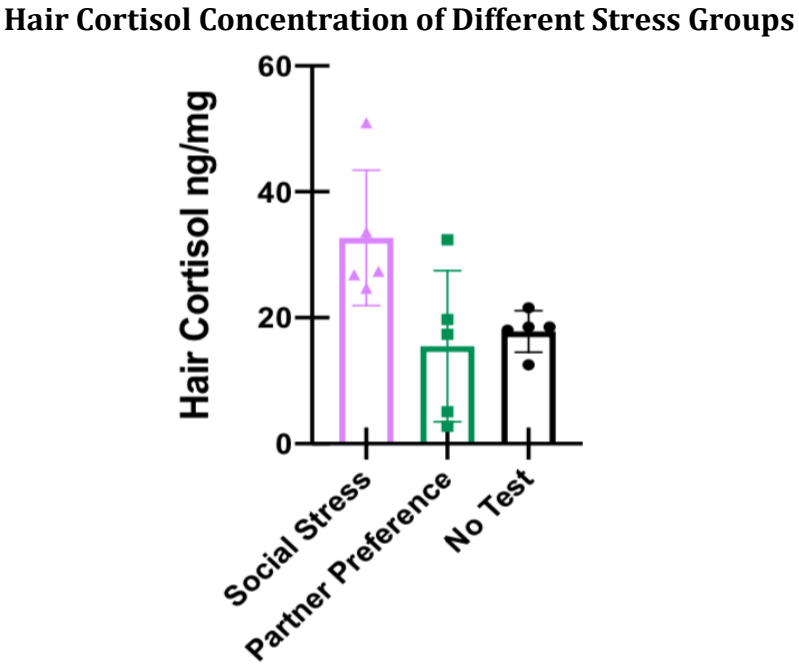

Figure 1. Hair *cortisol* concentration variation of different stress groups (n=5,5,5) according to the Kruskal-Wallis test.

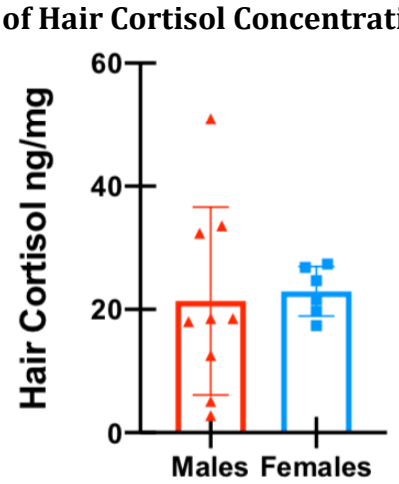

Figure 2. Hair *cortisol* concentration comparison of male and female marmosets (*n*=7,8) according to the Mann-Whitney U test.

Discussion

The serum assays did not yield any concentration of the biomarkers *IL-6*, *CRP*, *IGF-1* and *TNF-α*. One purpose of this experiment was to validate these biomarkers in the marmoset model, which was unsuccessful. One possible reason for the lack of antibody concentration in the assay plates could be because the assays purchased were for monkeys and did not specific marmosets. It is possible that biomarkers *IL-6*, *CRP*, *IGF-1* and *TNF-α* require species-specific antibodies.

There is a significant difference between the control group and the high stress experimental group. There is no significant difference between the control and low stress experimental group, or between the high stress and low stress experimental groups. This provides evidence that hair *cortisol* is a reliable biomarker for long-term chronic stress as measured by hair samples. Hair *cortisol* is non-invasive and a relatively simple method of quantifying long-term changes in the physiological changes in stress and HPA function in marmosets.

Immunomarkers *TNF-α, IL-6, CRP,* and *IGF-1* were not present in marmoset serum indicated by the assay antibodies. However, this does not eliminate the possibility of marmosets utilizing these biomarkers. Although the antibodies for biomarkers *TNF-α, IL-6, CRP,* and *IGF-1* seem to require species-specificity, the antibodies for *cortisol* does not. *Cortisol* is the main glucocorticoid in many mammals because it is evolutionarily necessary. Stress activates the HPA axis and causes the "fight-or-flight" response, which has evolved in many mammals as a survival mechanism in life-threatening situations. Although the other biomarkers also have important functions in humans, *cortisol* has a broader evolutionary context in many mammals.

A possible future direction is to retest marmoset serum using an assay kit specifically designed for marmoset antibodies. Even with concentration levels from the biomarkers, it is difficult to establish what is a high or low concentration. To eliminate this limitation, further research is needed on the baseline of the biomarker concentrations, as well as how the biomarker concentration increases or decreases when introduced to a stressor.

Hair samples were collected from both adults and juvenile marmosets for the hair *cortisol* concentration. Baseline may vary between adults and juveniles, so a possible future direction can be to compare the baseline between age groups. Although this research is preliminary, it provided evidence that marmosets can be used as an animal model to further understand and study stress levels relating to *cortisol*. Both acute levels from blood or urine and chronic levels from hair can be used to for stress-related studies translating to how humans are impacted by stressors due to social relationships.

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