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Synthetic cannabinoids severely elevate amino transferase levels. Natural cannabidiol does not.

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Abstract:

Cannabidiol (CBD) is a promising and well-studied medicinal compound found in cannabis. While CBD has a favorable safety profile, the deleterious health effects of synthetic cannabinoids are well documented. The human body is not equipped with the tools needed to catabolize synthetic cannabinoids. Among the enzymes recruited to removing them from the body are Alanine Amino Transferase (ALT) and Aspartate Amino Transferase (AST). The present article is broken into one naturalistic medical observation, and two studies. Each of these is concerned with the ALT and AST levels of patients exposed to cannabinoids. The medical observation is of four patients who mistakenly consumed a dangerous synthetic cannabinoid, JWH-018. Their ALT and AST levels were recorded once. The first experimental study is of six patients that consumed a synthetic CBD derivative, H_4-CBD. ALT and AST levels were recorded over 22 weeks. The second experimental study is of 184 patients that consumed natural CBD. ALT and AST levels were recorded over 6 months. Taken together, these studies demonstrate clear differences between consumption of natural CBD, and two synthetic derivatives on ALT and AST levels.

Cannabidiol (CBD) is a promising and well-studied medicinal compound found in cannabis. It has been shown to be anxiolytic, antidepressant, antipsychotic, anticonvulsant, anti-nausea, antioxidant, anti-inflammatory, anti-arthritic, anti-neoplastic, and protective in animal models of epilepsy, anxiety, psychosis, and basal ganglia diseases (Ligresti, De Petrocellis, & Di Marzo, 2016). Anti-cancer effects have also been shown (Pisanti et al., 2017). Research involving CBD has burgeoned in recent years (Burstein, 2015; Zuardi, 2008). Concurrently, there has been a proliferation of synthetic CBD derivatives (for a list, see Morales, Reggio, & Jagerovic, 2017). The World Drug Report 2016 showed that the majority of substances reported for the first time between 2012 and 2014 were synthetic cannabinoids.

While CBD has a favorable safety profile (Iffland, & Grotenhermen, 2017; Devinsky et al., 2016), the deleterious health effects of synthetic cannabinoids are well documented (van Amsterdam, Brung, & van den Brink, 2015; Law, Schier, Martin, Chang, Wolkin, & Schauben, 2016). Reported side effects of synthetic cannabinoid use include extreme anxiety, confusion, hallucinations and paranoia, violent behavior, suicidal thoughts, tachycardia, nausea, and vomiting. Unlike CBD, synthetic cannabinoids are extremely potent, full agonists of the cannabinoid receptors (Weinstein, Rosca, Fattore, & London, 2017; Spaderna, Addy, D'Souza, 2013; Fantegrossi, Moran, Radominska-Pandya, & Prather, 2014).

The human body is not equipped with the tools needed to catabolize synthetic cannabinoids. Among the enzymes recruited to remove them from the body are Alanine Amino Transferase (ALT) and Aspartate Amino Transferase (AST). In a normal, healthy body, ALT levels are typically reported between 7 and 56 units/L, and AST levels between 5 and 40. Severely heightened ALT and AST levels can result in liver failure and subsequently death (Robles-Diaz et al., 2014).

The present article is broken into one naturalistic medical observation, and two experimental studies. Each of these is concerned with the ALT and AST levels of patients exposed to cannabinoids. The medical observation is of four patients who mistakenly consumed a dangerous synthetic cannabinoid, most likely JWH-018. Their ALT and AST levels were recorded once. The first experimental study is of six patients that consumed a synthetic CBD derivative, H_4-CBD. ALT and AST levels were recorded over 22 weeks. The second experimental study is of 184 patients that consumed natural CBD. ALT and AST levels were recorded over 6 months. Taken together, these studies demonstrate clear differences between consumption of natural CBD, and two synthetic derivatives on ALT and AST levels.

Medical Observation: JWH-018

On December 21, 2016, an emergency response call was made from Morjin Beach, Goa, India to Healthway Hosptial. Four Russian tourists (men = 3, women = 1) were received in an agitated and diaphoretic state. They complained of headache, dizziness, and diaphoresis. They demonstrated a tachycardia average of 132 bpm. Otherwise they had normal vital signs and normal oxygenation.

The three males were uncooperative, restless, and aggressive in the emergency room. They were treated with 2 mg of lorazepam intravaneously. These patients improved after 10 hours and were kept for observation overnight.

The lone female was catatonic and lying supine with her eyes wide open. She was silent, and unresponsive to verbal commands and stimuli. She also displayed tachycardia of 135. Vertical nystagmus was noted. Examinations of her heart, lungs, and abdomen seemed normal. Her lower extremities were slightly bent but rigid. She was first treated with a dose of diphenhydramine (50 mg IV), which improved her rigid extremities and partially gave her the ability to speak. She was then treated with lorazepam and continued to improve. She was kept overnight. By morning, her motor and verbal skills returned to normal, and she was released.

All four erroneously reported smoking CBD, which one of the males stated he purchased from China. The standard urine toxicology screen was negative. Serum ethanol levels were normal. Comprehensive Metabolic Panel blood tests showed extremely high ALT levels (M = 1227.5 Units/L, SD = 178.88) and AST levels (M = 1136.75 Units/L, SD = 183.74) (See Table 1).

	ALT Levels	AST Levels
Man #1	1447	1233
Man #2	1233	1012
Man #3	1221	1346
Woman #1	1009	956

Table 1: Observed ALT and AST levels (Units/L) of four patients who consumed a synthetic cannabinoid, most likely JWH-018.

Urine samples were sent for further toxicological studies. Potential metabolites were analyzed in the urine samples collected by liquid chromatography-mass spectrometry (LC-MS/MS). Tests detected an average of 10.6 ng/ml of pentatonic acid. This is the predominant metabolite of JWH-018. Other metabolites detected were 5-and 4-HO pentyl-JWH-018 and JWH-073 butanoic acid. Occasionally, further hydroxylated metabolites were found.

Experimental Study 1: H_4-CBD

Six college students allegedly purchased 1 kg of what they believed to be CBD from MBI Import and Export Pvt Ltd, China. They brought the product to ImmunAG, LLP, Goa, India to verify its authenticity.

A CBD isolate from ImmunAG™ (Cushing, Kristipati, Shastri, & Joseph, 2018; Cushing & Joseph, 2018) was used as a reference standard against which the 1 kg sample was tested. The purity of the reference standard was confirmed by liquid chromatography–ultraviolet-mass spectrometry (LC–UV-MS) measurements. It had a bioactivity of .96, whereas the reference sample had a very low bioactivity of .06. This strikingly low bioactivity suggested that this product may not have been natural CBD.

Mass Isolation Vibrational Spectroscopy was subsequently used to analyze the sample. It was then identified as H_4-CBD: A synthetic, hydrogenated derivative of CBD.

The students were informed that the product they bought was a hydrogenated derivative and not natural CBD. They were told that the safety of the product was unknown. They decided to use the product anyways, but they agreed to have their health monitored while they used it.

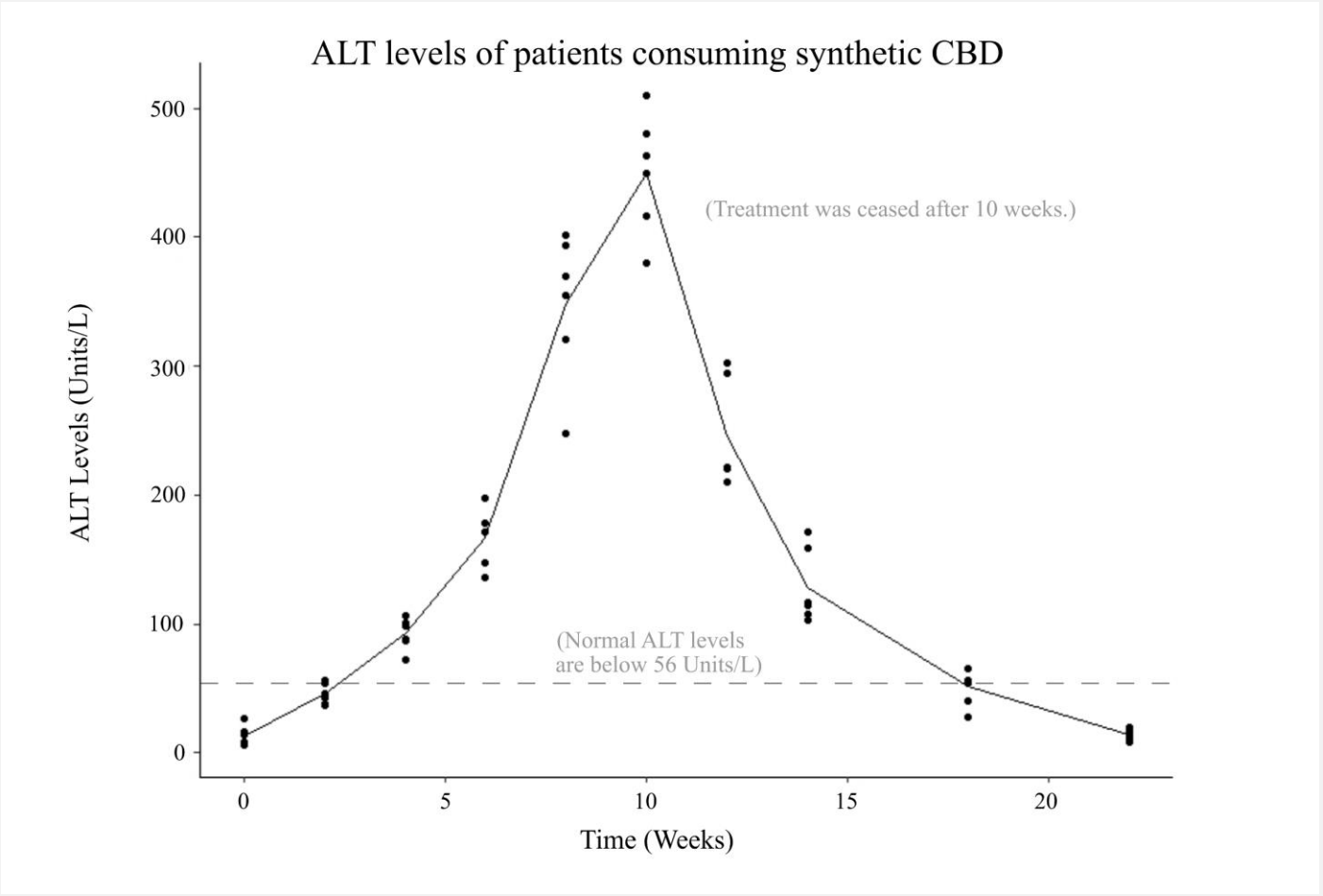

Figure 1: ALT levels increased drastically during H_4-CBD consumption, and returned to normal when patients ceased ingestion.

Method:

Subjects:

6 patients (men = 4, women = 2) consumed H_4-CBD, in 50 mg packets with variable total doses (*M* = 891.67 mg, *SD* = 58.45). Full data for H_4-CBD trials are listed in Appendix 1.

Procedure:

Every two weeks, patients had blood samples drawn at Healthway Hospital. AST and ALT levels were recorded. After 56 days, we strongly recommended to the patients that they stop using the product. After 70 days, patients stopped ingesting the H_4-CBD. AST and ALT levels were additionally examined 14, 28, 56, and 84 days after cessation.

Statistical analysis:

Growth curve modelling, as outlined in Field, Miles, and Field, 2012, would be an ideal way to analyze data of this sort, but ethical constraints limited the number of patients who could be exposed to the product in question. Instead, descriptive statistics, including means and standard deviations are provided for each time point. All AST and ALT levels are described in units/liter.

Results:

ALT levels at baseline ranged from 6 to 27 (*M* = 13.33, *SD* = 7.92). After 14 days of consumption, ALT levels ranged from 37 to 56 (*M* = 45.67, *SD* = 7.97). After 28 days, ALT levels ranged from 72 to 107 (*M* = 92.33, *SD* = 12.61). After 42 days, ALT levels ranged from 136 to 198 (*M* = 166.83, *SD* = 22.25). After 56 days, ALT levels ranged from 248 to 402 (*M* = 348.33, *SD* = 57.08). After 70 days, ALT levels ranged from 380 to 510 (*M* = 449.83, *SD* = 46.31). 14 days after cessation, ALT levels ranged from 210 to 302 (*M* = 245.33, *SD* = 41.49). 28 days after cessation, ALT levels ranged from 103 to 171 (*M* = 128.67, *SD* = 28.81). 56 days after cessation, ALT

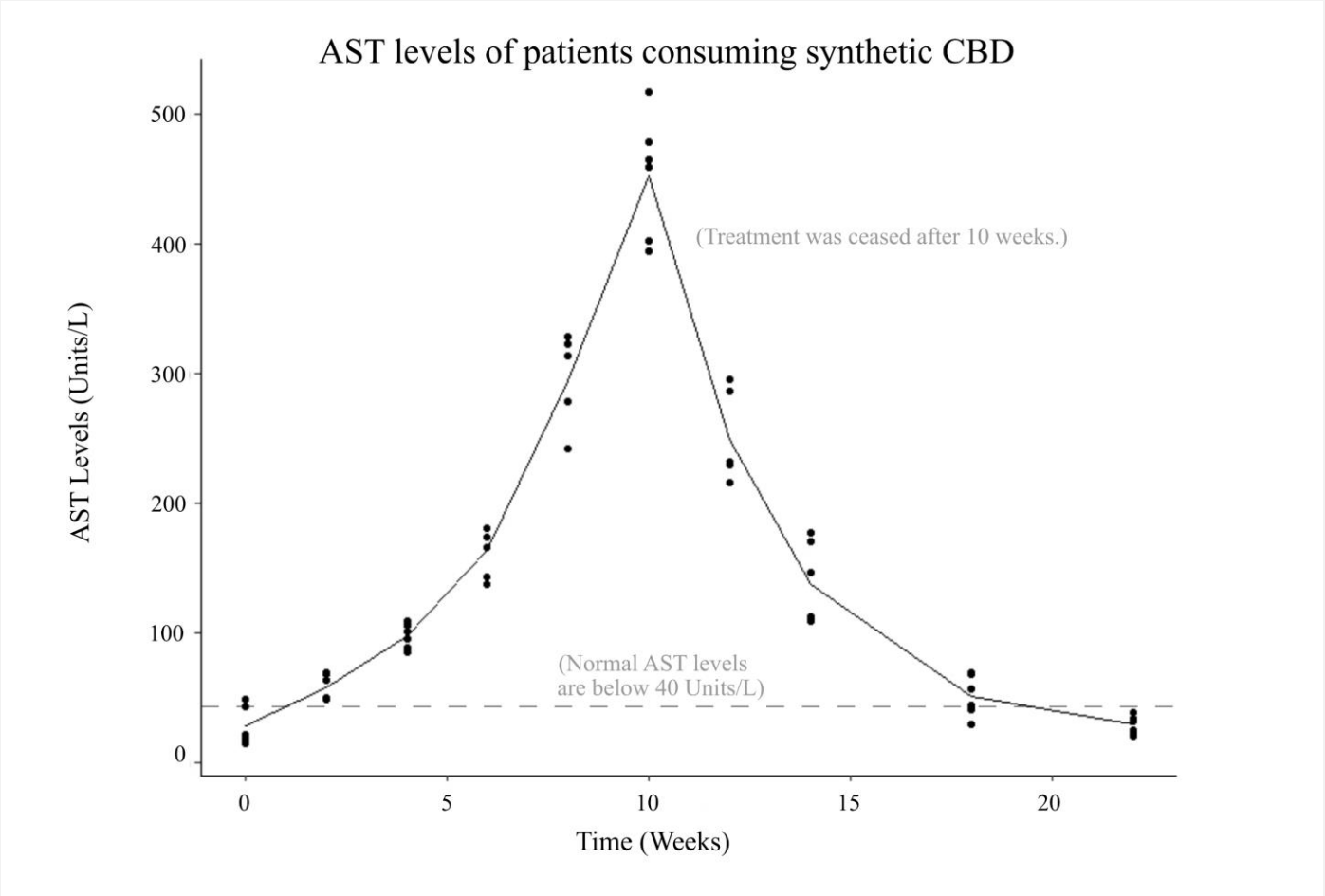

Figure 2: AST levels increased drastically during H_4-CBD consumption, and returned to normal when patients ceased ingestion.

levels ranged from 28 to 66 (*M* = 51.67, *SD* = 15.04). 84 days after cessation, ALT levels ranged from 8 to 20 (*M* = 13.83, *SD* = 5.04).

AST levels at baseline ranged from 14 to 48 (*M* = 27.33, *SD* = 14.36). After 14 days of consumption, AST levels ranged from 48 to 69 (*M* = 58, *SD* = 9.74). After 28 days, AST levels ranged from 85 to 109 (*M* = 97.17, *SD* = 9.52). After 42 days, AST levels ranged from 137 to 180 (*M* = 163.17, *SD* = 18.86). After 56 days, AST levels ranged from 242 to 328 (*M* = 293.5, *SD* = 33.21). After 70 days, AST levels ranged from 394 to 517 (*M* = 452.5, *SD* = 46.86). 14 days after cessation, AST levels ranged from 216 to 295 (*M* = 248, *SD* = 33.52). 28 days after cessation, AST levels ranged from 109 to 177 (*M* = 137.17, *SD* = 31.51). 56 days after cessation, AST levels ranged from 29 to 69 (*M* = 51, *SD* = 16.07). 84 days after cessation, AST levels ranged from 20 to 38 (*M* = 28.5, *SD* = 6.95).

To summarize, ALT and AST levels increased dramatically while patients were ingesting H_4-CBD. These levels returned to baseline 84 days after ceasing to ingest H_4-CBD (see Figures 1 & 2).

Experimental Study 2: Natural CBD

Having seen the toxicity of JWH-018 and H_4-CBD, we decided to study the safety of CBD. Volunteers were recruited through posters displayed in local hospitals throughout North Goa, India. Inclusion criteria are included in appendix 2. Volunteers were paid for their participation.

The CBD used in this study was an isolate acquired from ImmunAG, LLP (Cushing, Kristipati, Shastri, & Joseph, 2018).

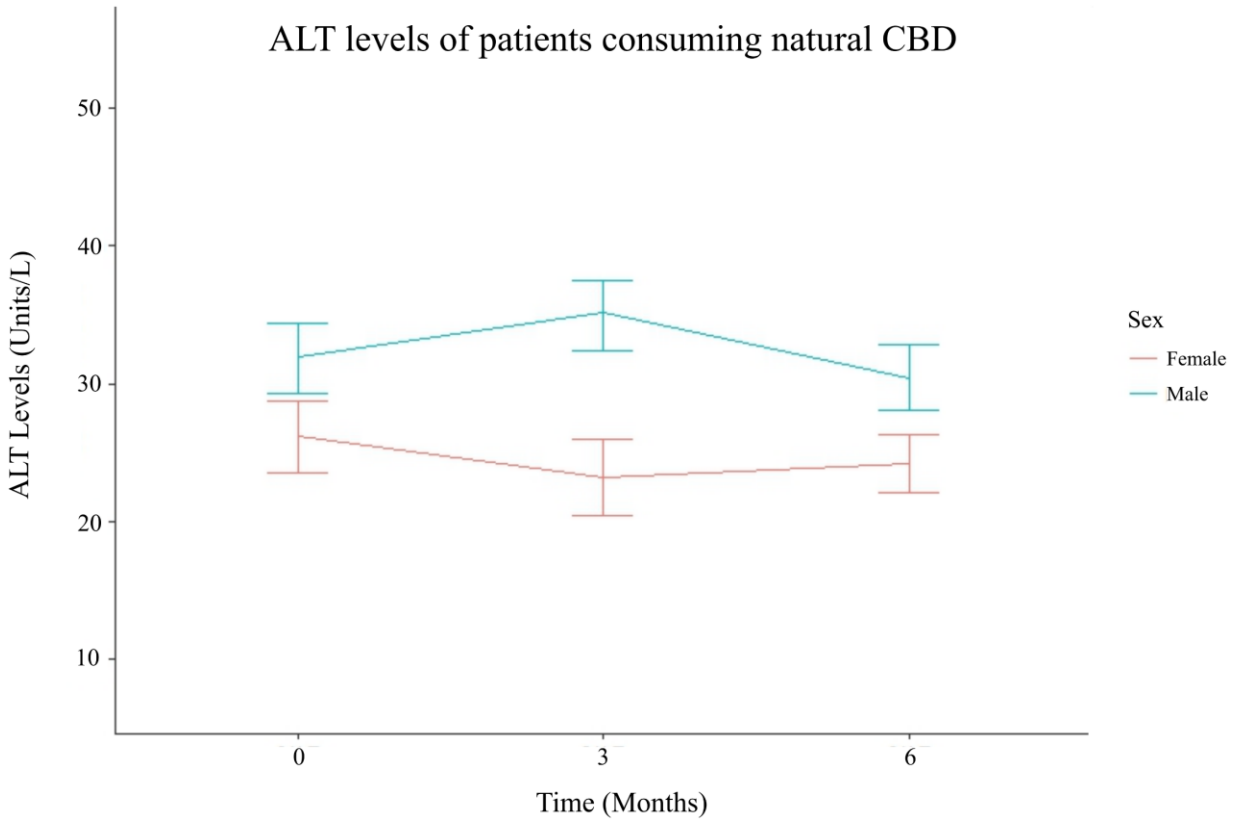

Figure 3: An increase in ALT levels among patients consuming natural CBD was not observed.

Method:

Subjects:

184 patients (men = 132, women = 56) consumed CBD isolated from ImmunAG, with variable dosages (*M* = 771.01 mg, *SD* = 264.82 mg). These dosages were recommended according to patient height (*M* = 11.09 mg/inch, *SD* = 4.05 mg/inch). CBD came in pill form, and patients were instructed consume it orally once per day.

Procedure:

AST and ALT levels were collected at the start of the experiment, after 3 months, and after 6 months via blood draw at Healthway Hospital, Goa, India.

Statistical analysis:

Repeated Measures ANOVAs were utilized to determine whether there were differences in AST or ALT levels over the 6-month consumption period, and whether there was an interaction effect of sex. ANOVAs were calculated using R version 3.4.4. Type III sums of squares were calculated through use of the 'ez' package. Descriptive statistics, including means and standard deviations are provided for each time point. All AST and ALT levels are described in units/liter. Full data for each patient, including height, dosage, dosage per height, and sex, are listed in Appendix 3.

Results:

Male AST levels at baseline ranged from 10 to 40 (*M* = 23.34, *SD* = 8.8). Female AST levels at baseline ranged from 9 to 56 (*M* = 31.25, *SD* = 13.02). Male AST levels at 3 months ranged from 12 to 44 (*M* = 29.01, *SD* = 9.55). Female AST levels at 3 months ranged from 9 to 56 (*M* = 31.57, *SD* = 14.72). Male AST levels at 6 months ranged from 10 to 40 (*M* = 25.59, *SD* = 8.83). Female AST levels at 6 months ranged from 8 to 55 (*M* = 29, *SD* = 14.36). Mean AST levels across the three time points are shown in Figure 3.

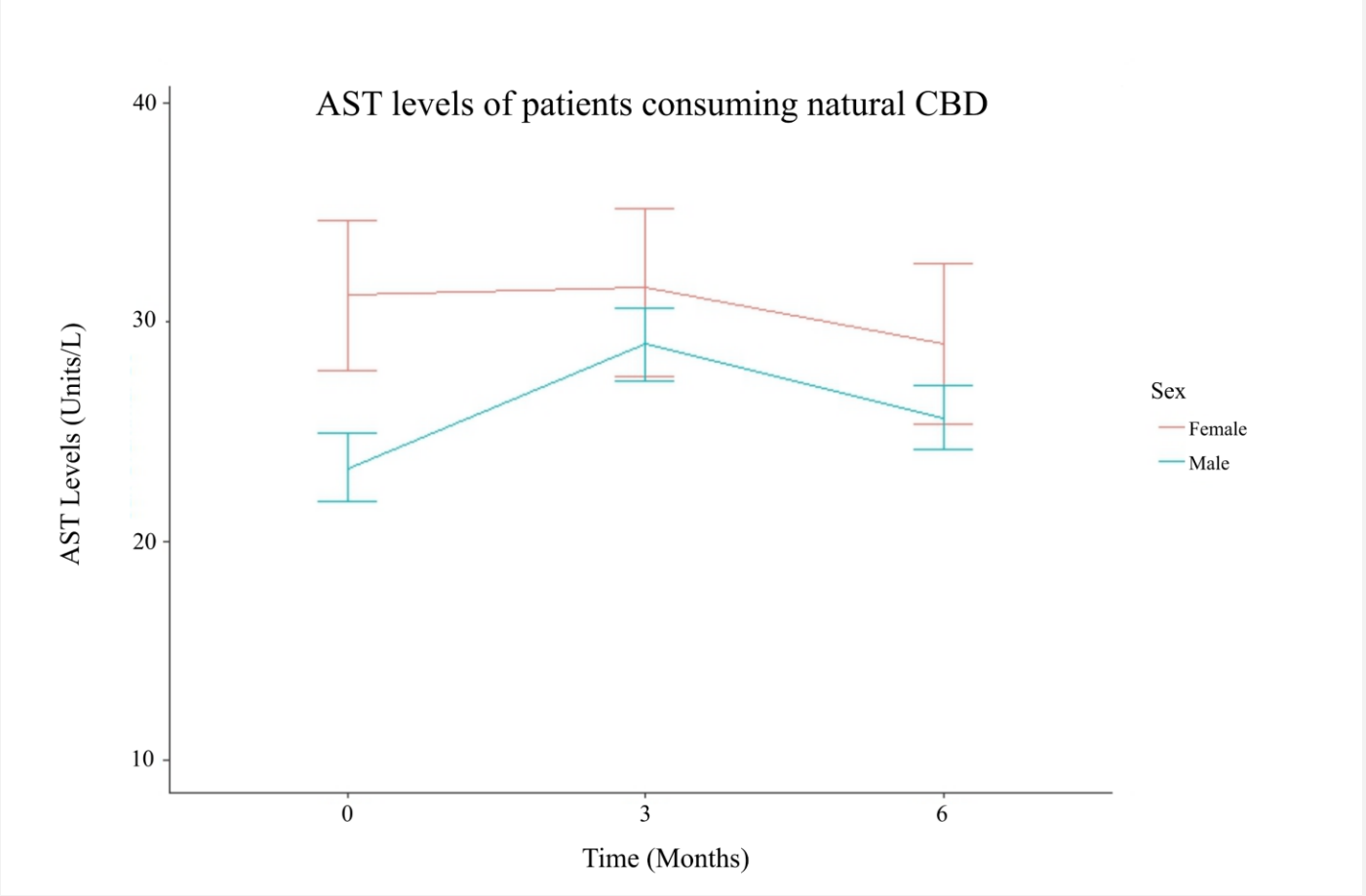

Figure 4: An increase in AST levels among patients consuming natural CBD was not observed.

A 2x3 Repeated Measures ANOVA was conducted. Mauchly's test for sphericity revealed no violation for the repeated measures variable, $W = .99$, $p = .88$, or for the interaction, $W = .99$, $p = .88$. There was a significant main effect of sex, $F(1, 186) = 19.75$, $p < .001$ Generalized $\eta^2 = .04$, and of timepoint, $F(2, 372) = 5.79$, $p = .003$, Generalized $\eta^2 = .02$. There was not a significant interaction effect, $F(2, 372) = 2.94$, $p = .054$.

Bonferroni-corrected post-hoc comparison revealed a significant difference between baseline levels and levels at 3 months among Males, $p = .004$. No other comparisons were significant (every $p > .15$).

Male ALT levels at baseline ranged from 7 to 56 ($M = 31.9$, $SD = 14.51$). Female ALT levels at baseline ranged from 8 to 40 ($M = 26.18$, $SD = 9.86$). Male ALT levels at 3 months ranged from 10 to 60 ($M = 35.18$, $SD = 15.18$). Female ALT levels at 3 months ranged from 8 to 40 ($M = 23.14$, $SD = 10.38$). Male ALT levels at 6 months ranged from 8 to 56 ($M = 30.45$, $SD = 14.48$). Female ALT levels at 6 months ranged from 8 to 39 ($M = 24.16$, $SD = 8.54$). Mean ALT levels across the three time points are shown in Figure 4.

A 2x3 Repeated Measures ANOVA was conducted. Mauchly's test for sphericity revealed no violation for the repeated measures variable, $W = .98$, $p = .19$, or for the interaction, $W = .98$, $p = .19$. There was a significant main effect of sex, $F(1, 186) = 41.47$, $p < .001$ Generalized $\eta^2 = .07$. There was a significant main effect of timepoint, $F(2, 372) = 3.94$, $p = .02$, Generalized $\eta^2 = .01$. There was not a significant interaction effect, $F(2, 372) = 2.70$, $p = .07$.

Bonferroni-corrected post-hoc comparison revealed no significant differences. However, among Males, a potential difference between 3 months and 6 months approached significance, $p = .06$. No other comparisons were significant (every $p > .71$).

To summarize, AST and ALT levels remained within acceptable limits over the course of six months. There was no evidence of AST or ALT level elevation. Both potentially significant differences between

timepoints for either AST or ALT appeared among men. These differences were small, occurred between different timepoints, and did not point to an overall trend. As such, it is likely that these observed differences were produced by random chance.

Discussion:

This investigation began with the initial observation of ALT and AST levels 20 times higher than normal in four patients who mistakenly smoked JWH-018. These alarmingly high levels prompted our examination of another synthetic cannabinoid closer to the structure of CBD, H_4-CBD. Within 10 weeks, AST and ALT levels in six patients were observed to rise to 10 times the normal, and they returned to normal twelve weeks after patients stopped consuming it. By contrast, ALT and AST levels remained within the normal range over the course of six months for patients consuming natural CBD. In fact, none of these 184 patients had ALT or AST levels rise above 60.

Our results are in line with the observation that synthetic cannabinoids appear to pose a greater risk to users' health than natural cannabinoids (Winstock, Lynskey, Borschmann, & Waldron, 2015). The clear differences outlined here should serve as a warning to patients and practitioners alike that synthetic variants of CBD should not be blindly substituted for natural CBD.

References:

Burstein, S. (2015). Cannabidiol (CBD) and its analogs: a review of their effects on inflammation. *Bioorganic & medicinal chemistry*, *23*(7), 1377-1385. https://doi.org/10.1016/j.bmc.2015.01.059

Cushing, D., & Joseph, B. (2018). Identification of cannabidiol from Humulus Kriya using x-ray crystallography. *Journal of Medical Phyto Research*, 29. https://doi.org/10.31013/2002d

Cushing, D., Kristipati, S., Shastri, R., Joseph, B., & Newark, C. A. (2018). Measuring the bioactivity of phytocannabinoid cannabidiol from cannabis sources, and a novel non-cannabis source. *Journal of Medical Phyto Research*, 10. https://doi.org/10.31013/2002b

Devinsky, O., Marsh, E., Friedman, D., Thiele, E., Laux, L., Sullivan, J., ... & Wong, M. (2016). Cannabidiol in patients with treatment-resistant epilepsy: an open-label interventional trial. *The Lancet Neurology*, *15*(3), 270-278. https://doi.org/10.1016/s1474-4422(15)00379-8

Fantegrossi, W. E., Moran, J. H., Radominska-Pandya, A., & Prather, P. L. (2014). Distinct pharmacology and metabolism of K2 synthetic cannabinoids compared to Δ9-THC: mechanism underlying greater toxicity?. *Life sciences*, *97*(1), 45-54. https://doi.org/10.1016/j.lfs.2013.09.017

Iffland, K., & Grotenhermen, F. (2017). An Update on Safety and Side Effects of Cannabidiol: A Review of Clinical Data and Relevant Animal Studies. *Cannabis and cannabinoid research*, *2*(1), 139-154. https://doi.org/10.1089/can.2016.0034

Ligresti, A., De Petrocellis, L., & Di Marzo, V. (2016). From phytocannabinoids to cannabinoid receptors and endocannabinoids: pleiotropic physiological and pathological roles through complex pharmacology. *Physiological reviews*, *96*(4), 1593-1659. https://doi.org/10.1152/physrev.00002.2016

Morales, P., Reggio, P. H., & Jagerovic, N. (2017). An overview on medicinal chemistry of synthetic and natural derivatives of cannabidiol. *Frontiers in pharmacology*, *8*, 422. https://doi.org/10.3389/fphar.2017.00422

Pisanti, S., Malfitano, A. M., Ciaglia, E., Lamberti, A., Ranieri, R., Cuomo, G., ... & Laezza, C. (2017). Cannabidiol: State of the art and new challenges for therapeutic applications. *Pharmacology & therapeutics*, *175*, 133-150. https://doi.org/10.1016/j.pharmthera.2017.02.041

Robles–Diaz, M., Lucena, M. I., Kaplowitz, N., Stephens, C., Medina–Cáliz, I., González–Jimenez, A., ... & Jimenez–Perez, M. (2014). Use of Hy's law and a new composite algorithm to predict acute liver failure in patients with drug-induced liver injury. Gastroenterology, 147(1), 109-118. https://doi.org/10.1053/j.gastro.2014.11.046

Spaderna, M., Addy, P. H., & D'Souza, D. C. (2013). Spicing things up: synthetic cannabinoids. *Psychopharmacology*, *228*(4), 525-540. https://doi.org/10.1007/s00213-013-3188-4

van Amsterdam, J., Brunt, T., & van den Brink, W. (2015). The adverse health effects of synthetic

cannabinoids with emphasis on psychosis-like effects. *Journal of psychopharmacology*, *29*(3), 254-263. https://doi.org/10.1177/0269881114565142

Weinstein, A. M., Rosca, P., Fattore, L., & London, E. D. (2017). Synthetic Cathinone and Cannabinoid Designer Drugs Pose a Major Risk for Public Health. Frontiers in Psychiatry, 8, 156. https://doi.org/10.3389/fpsyt.2017.00156

Winstock AR, Lynskey M, Borschmann R, Waldron J. Risk of emergency medical treatment following consumption of cannabis or synthetic cannabinoids in a large global sample. J Psychopharmacol 2015. https://doi.org/10.1177/0269881115574493

Zuardi, A. W. (2008). Cannabidiol: from an inactive cannabinoid to a drug with wide spectrum of action. *Revista brasileira de psiquiatria*, *30*(3), 271-280. https://doi.org/10.1590/s1516-44462008000300015

Appendix 1: Full Data for H_4-CBD: Dangerous increase in AST/ALT over 10 weeks.

ID	27728	10688	12939	14195	25341	22991
Sex	M	M	M	F	M	F
mg/day	800	900	950	950	900	850
Initial ALT	48	14	21	18	20	43
2 week ALT	50	68	63	48	69	50
4 week ALT	109	88	85	101	95	105
6 week ALT	165	180	174	180	137	143
8 week ALT	313	278	328	278	242	322
10 week ALT	459	394	402	478	465	517
12 week ALT	295	216	229	232	286	230
14 week ALT	146	112	170	177	109	109
18 week ALT	29	40	44	56	68	69
22 week ALT	23	25	38	34	20	31
Initial AST	9	14	6	27	17	7
2 week AST	37	38	56	46	43	54
4 week AST	88	87	72	99	107	101
6 week AST	178	198	147	171	136	171
8 week AST	370	394	321	402	355	248
10 week AST	480	463	510	416	450	380
12 week AST	302	222	222	210	295	221
14 week AST	103	171	114	108	159	117
18 week AST	28	40	54	66	66	56
22 week AST	19	8	12	9	15	20

Appendix 2: Inclusion/Exclusion Criteria for H_4-CBD study:

Inclusion Criteria-
- Over the age of 18 at the time of screening.
- Judged by the study physician (D. Goakar) to be in generally good health.
- Body mass index between 18-35 kg/m2.
- Negative urine pregnancy test for women.

Exclusion Criteria-
- History of significant allergic condition, significant drug-related hypersensitivity, or allergic reaction to cannabinoids.
- Used cannabis, synthetic cannabinoid, cannabinoid analogue, or any CBD or THC-containing product within 30 days of eligibility screening.
- Patient has had a change in psychopharmacotherapy regimen in the last 4 weeks, or has any plans to change regimen over the course of the study.
- Current or past DSM-5 diagnosis of dissociative identity disorder, eating disorder with active purging, personality disorders, primary psychotic disorder, or bipolar affective disorder type 1.
- Patient is currently prescribed medications with possible CBD-drug interactions.
- History of actual suicide attempt in the last 5 years.
- Obstructive sleep apnea.
- Positive drug screen for THC, barbiturates, amphetamines, benzodiazepines, and/or opiates.
- History of treatment for, or evidence of, alcohol or drug abuse within the past year or regular alcohol consumption exceeding recommended limits.
- Lifetime history of Cannabis Use Disorder.

Appendix 3: Full Data for ImmunAG CBD; Null effects on ALT and AST levels

ID	Sex	mg/day	Initial ALT	3 month ALT	6 month ALT	Initial AST	3 month AST	6 month AST
3903084	M	1150	21	33	13	21	33	13
8414456	M	1200	38	16	31	38	16	31
7298908	M	800	32	59	26	32	59	26
9300266	M	1050	32	17	42	32	17	42
7149599	M	550	19	26	28	19	26	28
6387135	M	950	52	44	22	52	44	22
2525346	M	750	13	45	16	13	45	16
2063642	M	800	30	31	35	30	31	35
9282499	M	1000	23	35	23	23	35	23
5134362	M	1000	34	22	35	34	22	35
6942482	M	550	30	52	9	30	52	9
8047747	M	800	29	40	39	29	40	39
4902772	M	650	56	52	16	56	52	16
5543857	M	1000	9	52	53	9	52	53
7761964	M	850	23	35	22	23	35	22
7686663	M	350	16	56	55	16	56	55
9905916	M	400	12	52	47	12	52	47
8644764	M	450	56	21	50	56	21	50

5916306	M	750	54	43	16	54	43	16
6970960	M	800	17	16	20	17	16	20
5762713	M	850	47	31	56	47	31	56
3548060	M	550	32	37	40	32	37	40
2143482	M	700	50	37	15	50	37	15
9757661	M	800	10	42	19	10	42	19
1724204	M	1000	50	12	38	50	12	38
838415	M	350	25	49	51	25	49	51
2445581	M	850	54	34	48	54	34	48
7774440	M	700	28	10	19	28	10	19
5451833	M	800	48	42	8	48	42	8
5508098	M	1150	17	27	9	17	27	9
7296886	M	500	56	25	46	56	25	46
8692052	M	700	44	15	26	44	15	26
2595136	M	1000	35	50	39	35	50	39
1009593	M	850	24	23	50	24	23	50
8307501	M	300	39	23	18	39	23	18
7607767	M	1050	13	52	19	13	52	19
4757367	M	350	18	14	18	18	14	18
1144502	M	350	52	23	24	52	23	24
3668849	M	800	8	17	12	8	17	12
5731492	M	750	38	57	28	38	57	28
5013991	M	300	54	24	29	54	24	29
148354	M	1150	33	26	14	33	26	14
7089837	M	350	33	23	10	33	23	10
5137584	M	450	48	19	40	48	19	40
40356	M	1150	40	39	20	40	39	20
5583214	M	600	15	33	21	15	33	21
3345178	M	1000	47	19	49	47	19	49
1031842	M	1100	38	47	54	38	47	54
937758	M	600	26	33	23	26	33	23
9581523	M	1000	10	60	43	10	60	43
5497829	M	1000	25	42	40	25	42	40
2468790	M	300	24	53	13	24	53	13
8230503	M	500	42	57	41	42	57	41
1778564	M	1050	28	48	18	28	48	18
3921047	M	1100	21	52	14	21	52	14
3678708	M	400	29	47	40	29	47	40
5401913	M	400	20	18	55	20	18	55
6239329	M	700	37	52	10	37	52	10
8683910	M	1150	14	27	20	14	27	20
6458351	M	650	29	32	25	29	32	25
1910185	M	1150	51	42	23	51	42	23
7417283	M	1000	54	22	41	54	22	41
4714170	M	700	25	45	21	25	45	21
3406222	M	950	25	44	42	25	44	42
6000358	M	500	8	56	27	8	56	27
7008952	M	1100	38	48	35	38	48	35
5316453	M	1100	38	39	15	38	39	15

2924221	M	550	31	50	9	31	50	9
7680588	M	1100	31	39	47	31	39	47
8824091	M	850	20	28	8	20	28	8
2492940	M	850	48	11	43	48	11	43
8223432	M	1150	50	51	51	50	51	51
3237424	M	1150	39	38	55	39	38	55
3384779	M	350	41	29	29	41	29	29
474616	M	950	51	20	33	51	20	33
5611200	M	550	49	32	22	49	32	22
5650557	M	900	43	48	51	43	48	51
1233772	M	1000	10	19	33	10	19	33
4577950	M	850	16	57	19	16	57	19
5608793	M	400	12	49	43	12	49	43
6545551	M	1050	27	37	19	27	37	19
6127075	M	950	16	17	24	16	17	24
1624904	M	500	19	11	8	19	11	8
4092695	M	700	7	49	49	7	49	49
2323199	M	850	51	43	13	51	43	13
4100763	M	750	26	13	23	26	13	23
8020810	M	800	42	41	53	42	41	53
1699519	M	550	39	19	27	39	19	27
7100433	M	500	42	11	54	42	11	54
3339762	M	400	33	31	44	33	31	44
2023673	M	800	29	16	11	29	16	11
8481024	M	850	45	58	25	45	58	25
391209	M	550	33	14	38	33	14	38
7807493	M	800	13	39	44	13	39	44
2521663	M	1000	54	13	10	54	13	10
5926886	M	900	34	52	32	34	52	32
1927245	M	500	50	54	30	50	54	30
8935197	M	350	45	38	15	45	38	15
4251651	M	1000	51	14	12	51	14	12
7174872	M	400	15	57	8	15	57	8
4855461	M	300	46	37	41	46	37	41
3679552	M	800	33	14	18	33	14	18
6171493	M	1200	18	30	40	18	30	40
4394926	M	800	54	21	51	54	21	51
7631350	M	350	19	10	8	19	10	8
1016130	M	550	25	11	48	25	11	48
1489746	M	850	27	28	41	27	28	41
7099947	M	600	17	34	14	17	34	14
2750505	M	700	46	43	42	46	43	42
3983277	M	900	20	35	46	20	35	46
8560228	M	1050	53	22	44	53	22	44
4169021	M	900	40	12	43	40	12	43
714573	M	550	50	55	26	50	55	26
6840648	M	900	18	59	49	18	59	49
8464553	M	700	40	20	12	40	20	12
2557248	M	1050	15	32	34	15	32	34

4879447	M	550	26	55	15	26	55	15
8979211	M	750	56	54	49	56	54	49
7000022	M	900	18	58	35	18	58	35
8698541	M	800	23	14	15	23	14	15
5798974	M	1200	52	46	18	52	46	18
9137737	M	550	52	48	44	52	48	44
1161410	M	500	7	19	48	7	19	48
9641435	M	700	25	54	22	25	54	22
32644	M	300	18	39	34	18	39	34
7839138	M	800	53	55	55	53	55	55
360802	M	900	19	45	35	19	45	35
6286688	M	800	35	55	14	35	55	14
8212933	M	850	18	19	19	18	19	19
7148131	M	700	13	10	43	13	10	43
1399782	M	1150	11	45	32	11	45	32
8573654	M	750	16	53	38	16	53	38
1274	F	700	9	15	15	9	15	15
1365	F	500	19	23	16	19	23	16
1384	F	1000	36	20	25	36	20	25
1468	F	650	32	11	26	32	11	26
1487	F	350	15	39	26	15	39	26
1592	F	800	38	31	33	38	31	33
1594	F	550	21	10	24	21	10	24
2123	F	1150	39	24	26	39	24	26
2160	F	850	40	24	37	40	24	37
2169	F	1150	40	32	22	40	32	22
2390	F	700	35	21	10	35	21	10
2580	F	550	8	16	16	8	16	16
2634	F	950	37	17	35	37	17	35
2636	F	1000	19	26	33	19	26	33
2701	F	500	13	38	38	13	38	38
2712	F	350	36	14	27	36	14	27
2752	F	700	26	16	8	26	16	8
2905	F	850	38	11	37	38	11	37
3082	F	300	20	17	14	20	17	14
3249	F	1000	38	16	24	38	16	24
3331	F	750	18	24	35	18	24	35
3396	F	1150	28	38	26	28	38	26
3410	F	1000	31	9	32	31	9	32
3735	F	1000	33	37	24	33	37	24
4007	F	850	11	22	21	11	22	21
4015	F	800	38	9	23	38	9	23
4345	F	350	10	38	16	10	38	16
4392	F	450	30	30	9	30	30	9
4655	F	1100	15	9	37	15	9	37
4800	F	1000	24	29	31	24	29	31
4921	F	1150	35	32	13	35	32	13
4938	F	850	38	30	9	38	30	9
5213	F	600	27	28	32	27	28	32

5520	F	950	31	35	28	31	35	28
5753	F	1150	28	10	25	28	10	25
5835	F	1100	27	36	18	27	36	18
6143	F	1100	20	11	16	20	11	16
6312	F	600	25	40	15	25	40	15
6373	F	300	14	26	23	14	26	23
6381	F	1050	39	17	39	39	17	39
7112	F	1000	25	29	18	25	29	18
7178	F	350	23	39	21	23	39	21
7486	F	800	23	38	36	23	38	36
7650	F	350	29	13	26	29	13	26
7827	F	1100	13	8	29	13	8	29
7846	F	1150	24	31	36	24	31	36
7916	F	850	32	15	10	32	15	10
8177	F	400	37	32	18	37	32	18
8213	F	400	21	25	23	21	25	23
8431	F	500	35	32	15	35	32	15
8532	F	300	37	9	20	37	9	20
8561	F	700	14	17	22	14	17	22
8992	F	1100	8	40	31	8	40	31
9730	F	1050	31	13	26	31	13	26
9828	F	900	10	15	35	10	15	35
9887	F	1150	23	9	23	23	9	23
M		771	30.2	31.60	28.57	30.20	31.60	28.57
SD		264.82	13.53	14.95	13.29	13.53	14.95	13.29

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Higher bioactivity cannabidiol in greater concentration more greatly reduces valvular interstitial cell calcification

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Abstract:

Valvular interstitial cells (VICs) are integral to heart valve homeostasis and structural leaflet integrity. Aberrant calcification of VICs leads to dangerous diseases including calcific aortic valve disease. VIC calcification can be reduced through modulation of the MAPK/ERK cascade by selective antagonism of the CB2 receptor. This is a well-studied target of Cannabidiol (CBD). Recently, it has become increasingly understood that not all CBD samples have the same degree of bioactive potential (bioactivity). The present study seeks to determine whether levels of CBD bioactivity have different effects on VIC calcification reduction. VICs were isolated from porcine aortic valve leaflets, induced to calcify, and treated with CBD or left untreated. CBD of varying bioactivity was clustered into 8 different levels (.20, .30, .50, .60, .70, .80, .90, and .95.) by rounding. Concentrations of 5, 10, 25, 40, and 100 mg were examined. Means, standard deviations, minimum, and maximum calcification reduction values for each combination of bioactivity and mg concentration are provided. Two 1x5 repeated measures ANOVAs on mg concentrations, holding bioactivity at .20, and at .95 , respectively, were performed. A 2x2 robust mixed ANOVA confirmed an interaction between bioactivity and mg concentration (*psihat* = -36.5, $p < .001$). Calcification was most reduced by the .95 bioactivity, 100 mg concentration treatment ($M = 55\%$, $SD = 6.66\%$). These results indicate that bioactivity is of central importance when considering CBD as a treatment for VIC calcification reduction.

The valves of the human heart are supple, flow-regulating membranes within a complex multichambered pump (Mohler et al., 2001; Butcher, Simmons, & Warnock, 2008). Within these valves, among an extracellular matrix, exist valvular interstitial cells (VICs), which are integral to heart valve homeostasis and structural leaflet integrity (Hjortnaes et al., 2015; Taylor, Batten, Brand, Thomas, & Yacoub, 2003). Calcification of VICs causes disruption of interstitial cell mechanical phenotype, and drives disorganization of the interstitial matrix, nodule formation, and pro-calcific signaling (Farrar, Pramil, Richards, Mosher, & Butcher, 2016). This can lead to any number of dystrophic calcification diseases of the heart, which each can increase the risk for developing other cardiovascular diseases (e.g., valvular stenosis, see Michel & Dipchand, 2017; de Simone et al., 2010; Gerdts et al., 2015).

Novel therapeutic targets for cardiovascular calcification reduction include cannabinoid receptor 2 (CB2; Kaschina, 2016). CB_2 is present in osteoclasts, osteoblasts, and osteocytes (Ofek et al., 2006; Idris, Sophocleous, Landao-Bassonga, van't Hof, & Ralston, 2008; Idris et al., 2005). CB_2 is upregulated in calcifying heart valves (Naito et al., 2010). CB_2 agonists, such as anandamide, have been shown to stimulate extracellular signal-regulated kinase (ERK) activation (McAllister & Glass, 2002). Greater ERK activation yields greater calcific nodule formation in VICs (Gu & Masters, 2009). Thus, antagonism or inverse agonism of CB_2 should yield reductions in calcification.

Cannabidiol (CBD), the major nonpsychotropic component found in *Cannabis Sativa* (Ligresti Petrocellis, & Di Marzo, 2016) and recently, in a non-cannabis source (Kriya Brand Humulus; see Cushing, Kristipati, Shastri &

Concentration (mg)	Measure	Bioactivity							
		.20	**.30**	**.50**	**.60**	**.70**	**.80**	**.90**	**.95**
5	*M*	5.17	6.16	7.63	7.99	10.06	14.32	18.06	19.42
	SD	0.83	0.54	0.88	0.85	1.28	1.35	1.22	1.8
	Min	3.8	5.2	5.3	6.2	7.3	11.3	14.6	15.4
	Max	6.9	7.3	9.1	10.4	13.6	16.6	21	22.1
10	*M*	4.95	6.24	9.77	11.8	14.33	20.03	25.04	25.56
	SD	0.62	0.66	0.99	1.1	1.48	2.5	2.62	3.04
	Min	3.7	4.9	7.7	9.2	10.2	15.9	20.5	18.5
	Max	6.2	7.5	11.7	14.3	17.2	24.6	30.3	32.5
25	*M*	6.07	6.23	10.89	13.75	16.55	24.94	30.51	32.69
	SD	0.54	0.89	0.8	0.95	1.34	2.63	3.02	4.33
	Min	4.8	4.6	9.5	10.5	14.3	20.2	23.5	26.3
	Max	7.1	8.1	12.5	15.3	18.6	30.3	35.6	42.6
40	*M*	5.19	7.13	14.2	19.23	24.7	28.54	40.09	42.98
	SD	0.75	0.76	1.4	1.5	3.2	3.02	2.77	4.8
	Min	3.6	5.1	12	168	18.9	22.4	33.7	35.8
	Max	6.9	8.1	17.1	21.9	33	34.6	44.6	54.5
100	*M*	6.1	7.2	16.1	21.54	30.71	37.74	48.52	55
	SD	0.58	0.64	1.05	1.22	2.18	2.87	4.27	6.66
	Min	5.2	6	14.1	18.3	25.8	31.7	37.6	41.9
	Max	7.5	8.4	18.1	23.7	35.6	44.4	54.7	66.1

Table 1: Calcification reduction by CBD mg concentration and bioactivity. All calcification reductions are presented as percentages.

Joseph, 2018). CBD acts as an inverse agonist or antagonist at CB_2 (Thomas, Baillie, Phillips, Razdan, Ross, & Pertwee, 2007). CBD suppresses osteoclast formation but stimujlates osteoclast functioning through antagonism of G-coupled protein receptor 55 (GPR55; Whyte et al., 2009)[1].

The present study examines the effect that CBD samples of varying bioactivities (See Cushing, Kristipati, Shastri, & Joseph, 2018) and varying mg concentrations have on the calcification of porcine VICs in vitro. If VIC samples treated with CBD calcify less, this would implicate CBD as a potential prophylactic treatment for valvular calcification diseases. If CBD bioactivity impacts the magnitude of calcification reduction, this would re-emphasize the importance of verifying bioactivity levels prior to scientific experimentation. If higher bioactivity CBD corresponds to greater reductions in calcification, this would suggest that higher bioactivity CBD should be preferred by medical professionals.

Results:

Multiplate wells containing calcifying VICs from 27 different tissue samples were treated with CBD of 8 different bioactivities, and 5 different mg concentrations, or left untreated. Average calcification was computed using the number of nodules per well, and the average area per nodule. Average total nodule area for untreated wells was 3.27 mm^2 (*SD* = 0.32, *min* = 0.5, *max* = 4.0) per well. All reported percent reductions in calcification were computed by dividing average nodule area of treated wells from 3.27 mm^2. CBD samples were clustered according to their bioactivity levels around the values, .20, .30, .50, .60, .70, .80, .90, and .95. Mg dosages of 5, 10, 25, 40, and 100 were examined.

[1] It bears noting that valve calcification often occurs in tandem with inflammation. CBD has anti-inflammatory that likely owe to the dynamic regulation of multiple activity-dependent pathways, including TRPV1, TRPV2, and TRPA1 (Petrocellis et al., 2011; Qin et al., 2008; Bisogno et al., 2001; Costa et al., 2004), 5HT1α receptors (Pazos et al., 2013), glycine receptors (Ahrens et al., 2009), TRPM8 channels (De Petrocellis et al., 2011), T-type voltage-gated calcium channels (Ross, Napier, & Connor, 2008), G protein-coupled receptor GPR55 (Ross, 2009), and several others (For a comprehensive review, see French et al., 2017). These pathways may also directly, or indirectly, influence calcification (e.g., Whyte et al., 2009). CB_2 transduces many immunomodulatory and anti-inflammatory effects as well (Mukhopadhyay et al., 2010; Steffens et al., 2005).

Reduction in Calcification of VIC cells: Comparing bioactivites across concentrations

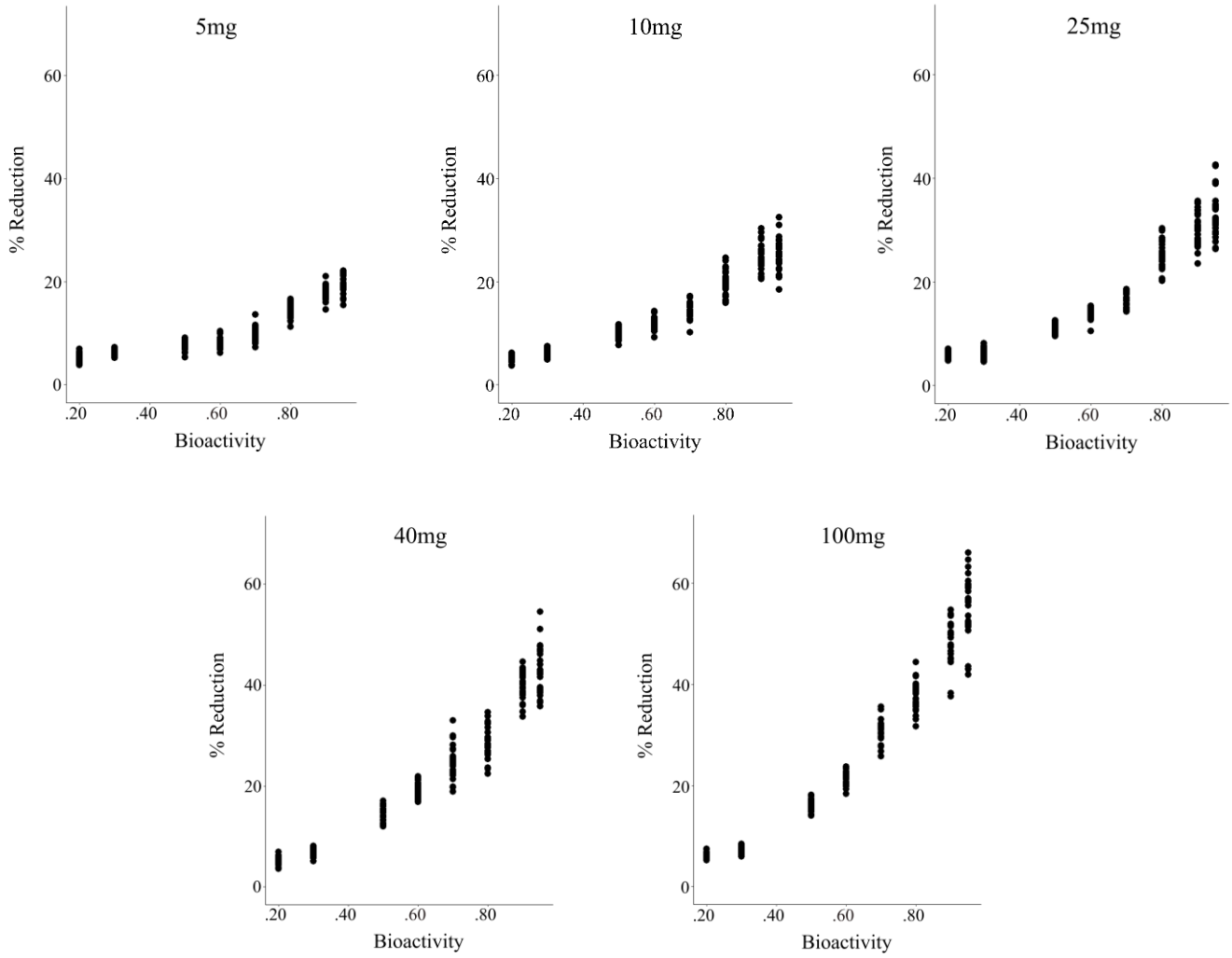

Figure 1: Across all five mg concentrations, there was greater calcification reduction amidst exposure to higher bioactivity CBD. Variance increased as reduction proportions came closer to .5.

Exploratory Data Analysis

Means, standard deviations, and minimum and maximum values for every tested bioactivity level at every tested mg concentration are provided in Table 1. (All data are provided in Appendix.) Higher bioactivities and higher concentrations each corresponded with reductions in calcification. At all five concentrations, with increases in bioactivity, calcification appeared to reduce exponentially (See Figure 1). Variance between the calcification reductions of each sample also increased as bioactivity increased. For example, the group with the highest reduction values (100 mg, .95 bioactivity) showed between 40% and 67% reduction (M = 55%). This group also had the greatest standard deviation (SD = 6.66%). Despite increased variability at higher reduction scores, meaningful differences appeared to exist between bioactivity levels. For example, for every mg concentration, the minimum calcification reduction elicited by .95 bioactivity CBD was still greater than the maximum calcification reduction elicited by .70 bioactivity CBD.

Comparing Low bioactivity CBD across mg concentrations

A 1x5 repeated measures ANOVA was conducted to examine whether differences existed between the various mg concentrations (5, 10, 25, 40, 100) among VIC samples treated with .20 bioactivity CBD (See Figure 2). Mauchley's Test found no violation to the assumption of

Reduction in Calcification of VIC cells: Comparing low and high concentrations across low and high bioactivities

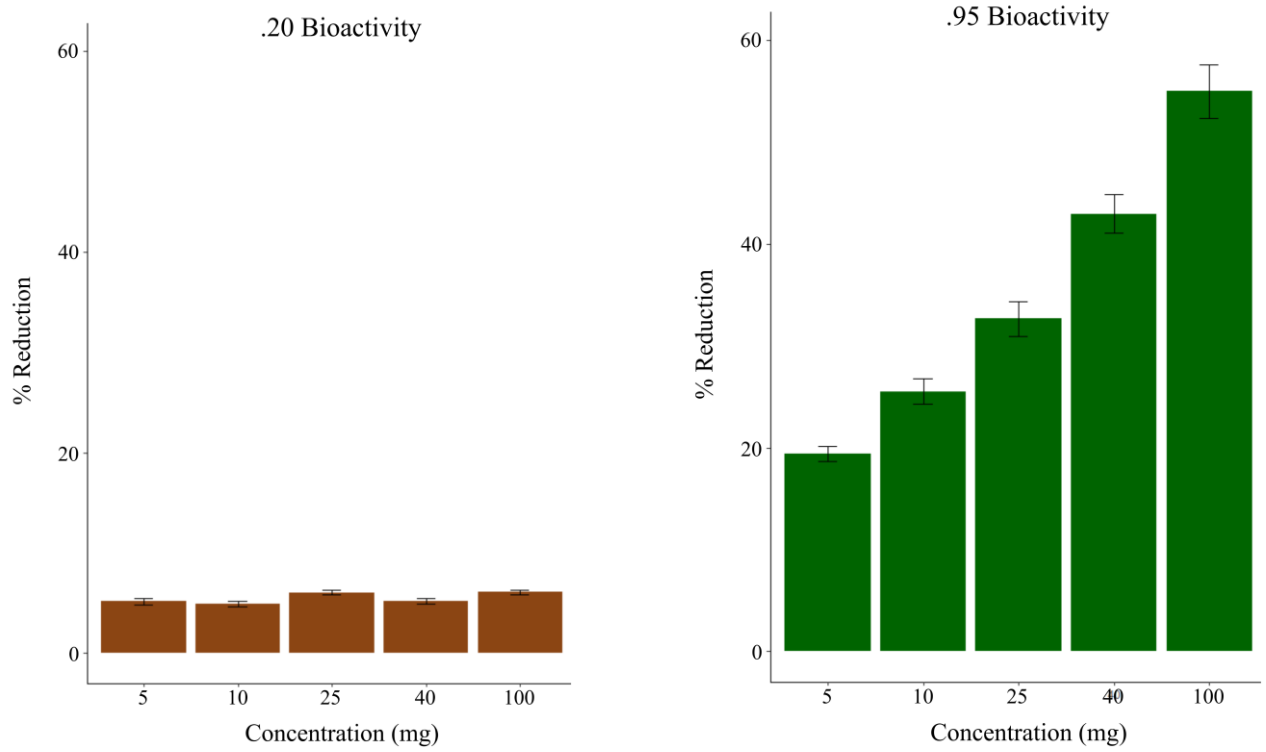

Figure 2: When bioactivity was very low, concentration made a negligible, inconsistent difference. When bioactivity was very high, higher concentrations yeilded greater calcification reductions.

sphericity between samples, $W = .759$, $p = .667$. The RM ANOVA found a significant difference among dosages on VIC calcification reduction within the .20 Bioactivity test group, $F(4, 104) = 18.90$, $p < .001$, $\eta^2_G = .354$. Post-hoc comparisons using the Bonferroni correction indicated that test samples exposed to dosages of 25 mg and 100 mg showed more reduction in VIC calcification than dosages 5, 10, and 40 mg. In all significant post-hoc comparisons, $p < .001$. The differences between these means were small (all $M_{diff} < 1.15\%$).

Comparing high bioactivity CBD across mg concentrations

A 1x5 repeated measures ANOVA was conducted to examine whether differences existed between the various mg concentrations among VIC samples treated with .95 bioactivity CBD (See Figure 2). Mauchley's Test found a possible violation to the assumption of sphericity, $W = .331$, $p = .0014$. A repeated measures ANOVA with a Greenhouse-Geisser correction found a significant difference among dosages on VIC calcification within the .95 bioactivity test group, $F(4, 104) = 260.13$, $p < .001$ $\eta^2_G = .894$. Post-hoc comparisons using the Bonferroni adjustment indicated significant differences across every group. In all significant post hoc comparisons, $p < .0000001$. The differences between these means were much larger (largest $M_{diff} = 35.58\%$).

Examining the interaction between bioactivity and concentration

Since there was an obvious trend across the data showing that higher bioactivity and concentration each lead to greater reductions in calcification, a 2x2 mixed ANOVA was attempted to better understand the degree to which bioactivity and dosage interact. Since the increase in calcification reduction appeared to occur similarly across all mg concentrations, we simplified our statistical approach by examining only the lowest and highest bioactivities and concentrations.

Levene's test showed heterogeneous variances, $W = 23.42$, $p < .001$. Arcsine transformation was unable to

render the variances homogeneous, $W = 16.04$, $p < .001$. Thus, a Robust 2x2 ANOVA using a median estimator was conducted on the original values. It found significant effects of bioactivity as a main effect (*psihat* = 64.7, $p < 0.001$), of concentration as a main effect (*psihat* = -37.5, $p < .001$), and of an interaction between bioactivity and concentration (*psihat* = -36.5, $p < .001$).

Conclusions

At high bioactivities and concentrations, calcification was reduced greatly. At lower bioactivities, mg concentration played an inconsistent role in calcification reduction. At high bioactivities, calcification reduction increased significantly at higher concentrations. A significant interaction effect was found between bioactivity and concentration through a robust estimation technique. Taken together, these results suggest that the bioactivity of the CBD is of central importance when considering the efficacy of CBD as a VIC calcification treatment.

Discussion:

In this study, CBD was shown to be effective at reducing porcine VIC calcification in vitro, with the highest bioactivity CBD at the highest concentration showing the greatest reductions ($M = 55\%$).

Within this experiment, we find ERK inhibition the most likely mechanism by which CBD chiefly reduced VIC calcification (see Gu & Masters, 2009). If this judgment is correct, then the differential calcification reduction caused by bioactivity level implies the differential inhibition of ERK activity. The implications of this are vast.

The ERK pathway links diverse extracellular stimuli to proliferation, differentiation, survival, and vascularization (Roy et al, 2001, Salasznyk et al, 2004; Lewis, Shapiro & Ahn, 1998; Depeille, 2007). A tremendous number of diseases can be affected through its modulation, including cancers (e.g., Wagner & Nebreda, 2009; Huang et al., 2008; Herrera, Carracedo, Diez-Zaera, Guzmán, & Velasco, 2005; Milella et al., 2001). For example, in conjunction with radiation, the MAPK p38 pathway was one of the main drivers of CBD-induced cell death in Glioblastoma (Ivanov et al., 2017). Additionally, the modulation by CBD of ERK and ROS pathways lead to the down-regulation of Id-1 expression and the up-regulation of Id-2, thereby inhibiting breast cancer cell proliferation and invasion (McAllister et al., 2010). The degree to which CBD elicits these anti-cancer effects would as well depend on the bioactivity of the CBD.

The bioactivity testing procedure used in this experiment, as described in Cushing et al. (2018), consisted of a monoclonal antibody test whose validity was based on the CB_2 affinity of CBD samples. It is as of yet unclear whether the CBD bioactivity test predicts the calcification reduction effects of non-CB_2 targets, such as GPR55 (Lauckner, Jensen, Chen, Lu, Hille, & Mackie, 2008). We expect that bioactivity generalizes to effects that are mediated through non-CB2 pathways. If so, research that utilized low bioactivity CBD to explore its pro-calcific effects on pathways such as GPR55 may have produced erroneous results. It is imperative that CBD samples be tested for bioactivity prior to clinical research.

Method:

All chemicals and solutions were obtained from Sigma- Aldrich (St. Louis, MO). All cell cultures were obtained from Creative Bioarray, Shirley, NY. CBD bioactivity was measured using practices described in Cushing, Kristipati, Shastri, and Joseph (2018).

VIC isolation and culture

VICs were isolated from porcine aortic valve leaflets (Hormel, Austin, MN) by collagenase digestion and subsequently cultured in growth medium (15% FBS, 2 mML-glutamine, 100 U/ml penicillin, and 100 g/ml streptomycin in medium 199) at 37°C, 5% CO2 for two to four passages. VICs used in all experiments were seeded at a density of 50,000 cells/cm^2 onto 24-well or 96-well plates. During the experiments, the VICs were cultured in low-serum medium (1% FBS, 100 U/ml penicillin, 100 g/ml streptomycin, 2 mM L-glutamine, in medium 199), and the medium was changed each day until the fifth day.

Culture substrate coatings

Tissue culture polystyrene (TCPS) plates (24-well or 96-well) were coated with type I collagen (Coll) (Inamed Biomaterials, Fremont, CA; 2 g/cm2), fibronectin (FN, 5 g/cm2), fibrin (FB, 1.5 g/cm2), or left untreated (TCPS). For the FB coating, plates were first incubated overnight at 4°C in fibrinogen (1 mg/ml), followed by three washes with 0.05% Tween 20 in phosphate- buffered saline (PBS) and 1 h incubation with thrombin (0.6 mg/ml) at 37°C (12). All coatings were prepared in 50 mM bicarbonate coating

buffer, pH 8.5, and rinsed three times with PBS before cell seeding. The amounts of adsorbed proteins were measured on separate plates using the bicinchoninic acid protein assay (Pierce, Rockford, IL) to verify adsorption of protein coatings.

MEK-1/2 inhibition

VICs exposed to various concentrations and bioactivities of CBD were treated with U-0126 [1,4-diamino- 2,3-dicyano-1,4-bis(2- aminophenylthio) butadiene; Calbiochem, San Diego, CA], PD-98059 (2-amino-3 methoxyflavone; 5 M; Calbiochem), or left untreated as a control to confirm the MAPK specificity of these inhibition experiments. U-0126 specifically inhibits MEK-1/2, thus inhibiting activation of ERK-1/2 (Favata et al., 1998). PD-98059 is an alternate MEK inhibitor. 9 tissue samples were in each treatment group. These were the tissue samples used in subsequent analyses.

Quantification of cell number

At time points of 1, 3, and 5 days, VICs were lysed with radioimmunoprecipitation assay buffer [1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 1 mM iodoacetamide, 140 mM NaCl, 10 mM Tris HCl, (pH 8.0)]. The amount of DNA in sample lysates was measured via the Quanti-iT PicoGreen assay (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions.

Migration assay

Migration was assayed via a modified fence method (Mann & West, 2002), wherein VICs were seeded within 2 mm^2 removable silicone wells, grown to confluency, and then allowed to migrate following the detachment of silicone isolators (defined as day 0). Grid-patterned transparencies were attached underneath plates containing VIC cultures to track cell movement over time. Photomicrographs were taken of the leading edge of cell migration under 40 magnification (Olympus IX51) every 24 h for 5 days. Net cell edge displacement was measured by overlaying time course images and then quantifying migration distance (NIH ImageJ) by measuring the advancement of the leading cell edge subtracted from the migration area recorded on day 0 within a single grid space.

Apoptosis assay

To ensure the health of the cell samples used in the calcification experiment, apoptosis was measured using an ELISA-based HT TiterTACS Assay Kit (Trevigen, Gaithersburg, MD), which detects DNA fragmentation. At days 1 and 5, cells were fixed in 3.7% buffered formaldehyde solution for 7 min, washed with PBS, and postfixed in 100% methanol for 20 min. Following manufacturer's instructions, the cells were permeabilized with proteinase K, quenched with 2.5% H2O2 in methanol, and then incubated with the labeling reaction mix (TdT, Biotin-dNTP, unlabeled dNTP) to label breaks in DNA. Streptavidin-HRP and then TACS-Sapphire were added to the wells to detect apoptotic cells; the reaction was stopped with 2 N HCl, and absorbance was read at 450 nm.

RNA isolation

Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH), according to the manufacturer's instructions. VICs were lysed with 200 l TRI Reagent per well at 4°C with 50 protease inhibitor cocktail (BD Biosciences, San Jose, CA). The homogenate was stored at room temperature for 5 min to complete the dissociation of nucleoprotein complexes, at which point 0.15 ml chloroform per 600 l TRI Reagent was added to the homogenate, followed by centrifugation at 13,000 g for 15 min. After centrifugation, RNA was precipitated from the upper aqueous phase by adding 0.3 ml isopropanol per 600 l TRI Reagent to the tubes and then centrifuged at 13,000 g for 8 min. After this centrifugation step, the RNA pellet was washed with 75% ethanol and centrifuged at 8,000 g for 5 min. The RNA pellet was air dried and dissolved in 75l H2O at 60°C for 15 min. RNA samples were stored at 20°C until subsequent use.

Quantitative real-time PCR analysis

Custom primers for various markers of cell contractility and osteogenic activity were obtained from Invitrogen (Carlsbad, CA) and are listed in Table 1. For cDNA construction, 250 ng of original RNA isolated from samples were reverse transcribed using iScript (Bio-Rad Laboratories, Hercules, CA) as per manufacturer's instructions. Samples were processed for real-time PCR analysis by combining 0.5 l of the cDNA construction, 5 M of primers, and SYBR Green SuperMix (Bio-Rad) in a 15-l reaction, as specified in the manufacturer's protocol. For thermo cycling, a standard protocol was used: PCR reactions were run over 40 cycles of denaturing at 95°C for

15 s and annealed at 60°C for 1 min; this was followed by a melting curve analysis for 80 cycles of 55°C 0.5°C/cycle, 10 s per cycle, to further confirm the purity of the final PCR products, with each condition performed in triplicate (iCycler iQ Real-Time PCR Instrument, Bio-Rad). A standard comparative threshold cycle (or CT) method was used to analyze the PCR data. The CT of all samples were first normalized to -actin as an internal control, and then the CT values for experimental samples were further normalized to the negative control (VICs on Coll, which represented a non CBD condition).

Quantification of nodule number and size

After 5 days of culture in the presence or absence of U-0126 or PD-98059, VIC cultures were stained with Alizarin Red S (ARS) to facilitate quantification of calcified nodules, as ARS stains mineralized deposits red. Cultures were fixed with 10% neutral buffered formalin, stored at 4°C overnight, and stained with a 2% solution of ARS in PBS. Positively stained nodules were manually counted under a microscope (Olympus IX51 with Hamamatsu 285 digital camera and Simple PCI digital imaging software; Compix, Imaging Systems, Cranberry Township, PA). Nodule size was measured using ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij/), and photomicrographs were captured under 40 and 100 magnifications.

CBD samples and bioactivity testing

CBD samples with bioactivities .20, .30, .50, .60, and .70 were obtained from Randy Kindred, Natural Hemp Solutions. CBD samples with bioactivities .80, .90, and .95 were isolated from ImmunAG, a humulus product of ImmunAG LLP. Following isolation, bioactivity was measured using procedures outlined in Cushing, Kristipati, Shastri, and Joseph (2018).

References:

Ahrens, J., Demir, R., Leuwer, M., De La Roche, J., Krampfl, K., Foadi, N., ... & Haeseler, G. (2009). The nonpsychotropic cannabinoid cannabidiol modulates and directly activates alpha-1 and alpha-1-Beta glycine receptor function. *Pharmacology*, *83*(4), 217-222. https://doi.org/10.1159/000201556

Bisogno, T., Hanuš, L., De Petrocellis, L., Tchilibon, S., Ponde, D. E., Brandi, I., ... & Di Marzo, V. (2001). Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. *British journal of pharmacology*, *134*(4), 845-852. https://doi.org/10.1038/sj.bjp.0704327

Butcher, J. T., Simmons, C. A., & Warnock, J. N. (2008). Mechanobiology of the aortic heart valve. *Journal of Heart Valve Disease*, *17*(1), 62. https://doi.org/10.1007/978-1-4939-5617-3_12

Costa, B., Giagnoni, G., Franke, C., Trovato, A. E., & Colleoni, M. (2004). Vanilloid TRPV1 receptor mediates the antihyperalgesic effect of the nonpsychoactive cannabinoid, cannabidiol, in a rat model of acute inflammation. *British journal of pharmacology*, *143*(2), 247-250. https://doi.org/10.1038/sj.bjp.0705920

Cushing, D., Kristipati, S., Shastri, R., & Joseph, B. (2018). Measuring the bioactivity of phytocannabinoid cannabidiol from cannabis sources, and a novel non-cannabis source. *Journal of Medical Phyto Research*, *10*. https://doi.org/10.31013/2002b

De Petrocellis, L., Ligresti, A., Moriello, A. S., Allarà, M., Bisogno, T., Petrosino, S., ... & Di Marzo, V. (2011). Effects of cannabinoids and cannabinoid-enriched Cannabis extracts on TRP channels and endocannabinoid metabolic enzymes. *British journal of pharmacology*, *163*(7), 1479-1494. https://doi.org/10.1111/j.1476-5381.2010.01166.x

de Simone, G., Izzo, R., Chinali, M., De Marco, M., Casalnuovo, G., Rozza, F., ... & De Luca, N. (2010). Does information on systolic and diastolic function improve prediction of a cardiovascular event by left ventricular hypertrophy in arterial hypertension?. *Hypertension*, *56*(1), 99-104. https://doi.org/10.1161/hypertensionaha.110.150128

Farrar, E. J., Pramil, V., Richards, J. M., Mosher, C. Z., & Butcher, J. T. (2016). Valve interstitial cell tensional homeostasis directs calcification and extracellular matrix remodeling processes via RhoA signaling. *Biomaterials*, *105*, 25-37. https://doi.org/10.1016/j.biomaterials.2016.07.034

Favata, M. F., Horiuchi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feeser, W. S., ... & Copeland,

R. A. (1998). Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *Journal of Biological Chemistry*, *273*(29), 18623-18632. https://doi.org/10.1074/jbc.273.29.18623

French, J. A., Koepp, M., Naegelin, Y., Vigevano, F., Auvin, S., Rho, J. M., ... & Dichter, M. A. (2017). Clinical studies and anti-inflammatory mechanisms of treatments. *Epilepsia*, *58*, 69-82. https://doi.org/10.1111/epi.13779

Gerdts, E., Rossebø, A. B., Pedersen, T. R., Cioffi, G., Lønnebakken, M. T., Cramariuc, D., ... & Devereux, R. B. (2015). Relation of left ventricular mass to prognosis in initially asymptomatic mild to moderate aortic valve stenosis. *Circulation: Cardiovascular Imaging*, *8*(11), e003644. https://doi.org/10.1161/circimaging.115.003644

Gu, X., & Masters, K. S. (2009). Role of the MAPK/ERK pathway in valvular interstitial cell calcification. *American Journal of Physiology-Heart and Circulatory Physiology*, *296*(6), H1748-H1757. https://doi.org/10.1152/ajpheart.00099.2009

Herrera, B., Carracedo, A., Diez-Zaera, M., Guzmán, M., & Velasco, G. (2005). p38 MAPK is involved in CB2 receptor-induced apoptosis of human leukaemia cells. *FEBS letters*, *579*(22), 5084-5088. https://doi.org/10.1016/j.febslet.2005.08.021

Hjortnaes, J., Shapero, K., Goettsch, C., Hutcheson, J. D., Keegan, J., Kluin, J., ... & Aikawa, E. (2015). Valvular interstitial cells suppress calcification of valvular endothelial cells. *Atherosclerosis*, *242*(1), 251-260. https://doi.org/10.1016/j.atherosclerosis.2015.07.008

Huang, D., Ding, Y., Luo, W. M., Bender, S., Qian, C. N., Kort, E., ... & Teh, B. T. (2008). Inhibition of MAPK kinase signaling pathways suppressed renal cell carcinoma growth and angiogenesis in vivo. *Cancer research*, *68*(1), 81-88. https://doi.org/10.1158/0008-5472.can-07-5311

Idris, A. I., Sophocleous, A., Landao-Bassonga, E., van't Hof, R. J., & Ralston, S. H. (2008). Regulation of bone mass, osteoclast function, and ovariectomy-induced bone loss by the type 2 cannabinoid receptor. *Endocrinology*, *149*(11), 5619-5626. https://doi.org/10.1210/en.2008-0150

Idris, A. I., van't Hof, R. J., Greig, I. R., Ridge, S. A., Baker, D., Ross, R. A., & Ralston, S. H. (2005). Regulation of bone mass, bone loss and osteoclast activity by cannabinoid receptors. *Nature medicine*, *11*(7), 774. https://doi.org/10.1038/nm1255

Kaschina, E. (2016). Cannabinoid CB1/CB2 Receptors in the Heart: Expression, Regulation, and Function. In *Cannabinoids in Health and Disease*. InTech. https://doi.org/10.5772/62822

Lauckner, J. E., Jensen, J. B., Chen, H. Y., Lu, H. C., Hille, B., & Mackie, K. (2008). GPR55 is a cannabinoid receptor that increases intracellular calcium and inhibits M current. *Proceedings of the National Academy of Sciences*, *105*(7), 2699-2704. https://doi.org/10.1073/pnas.0711278105

Lewis, T. S., Shapiro, P. S., & Ahn, N. G. (1998). Signal transduction through MAP kinase cascades. In *Advances in cancer research* (Vol. 74, pp. 49-139). Academic Press. https://doi.org/10.1016/s0065-230x(08)60765-4

Ligresti, A., De Petrocellis, L., & Di Marzo, V. (2016). From phytocannabinoids to cannabinoid receptors and endocannabinoids: pleiotropic physiological and pathological roles through complex pharmacology. Physiological reviews, 96(4), 1593- 1659. https://doi.org/10.1152/physrev.00002.2016

Mann, B. K., & West, J. L. (2002). Cell adhesion peptides alter smooth muscle cell adhesion, proliferation, migration, and matrix protein synthesis on modified surfaces and in polymer scaffolds. *Journal of biomedical materials research*, *60*(1), 86-93. https://doi.org/10.1002/jbm.10042

Michel, E. J. S., & Dipchand, A. I. (2017). Valvular Stenosis and Heart Failure. *Heart Failure in the Child and Young Adult: From Bench to Bedside*, 307. https://doi.org/10.1016/b978-0-12-802393-8.00023-5

Milella, M., Kornblau, S. M., Estrov, Z., Carter, B. Z., Lapillonne, H., Harris, D., ... & Andreeff, M. (2001). Therapeutic targeting of the MEK/MAPK signal transduction module in acute myeloid leukemia. *The Journal of clinical investigation*, *108*(6), 851-859. https://doi.org/10.1172/jci200112807

Mohler III, E. R., Gannon, F., Reynolds, C., Zimmerman, R., Keane, M. G., & Kaplan, F. S. (2001). Bone formation and inflammation in cardiac

valves. *Circulation*, *103*(11), 1522-1528. https://doi.org/10.1161/01.cir.103.11.1522

Mukhopadhyay, P., Rajesh, M., Pan, H., Patel, V., Mukhopadhyay, B., Bátkai, S., ... & Pacher, P. (2010). Cannabinoid-2 receptor limits inflammation, oxidative/nitrosative stress, and cell death in nephropathy. *Free Radical Biology and Medicine*, *48*(3), 457-467. https://doi.org/10.1016/j.freeradbiomed.2009.11.022

Naito, Y., Tsujino, T., Akahori, H., Ohyanagi, M., Mitsuno, M., Miyamoto, Y., & Masuyama, T. (2010). Augmented cannabinoid receptors expression in human aortic valve stenosis. *International journal of cardiology*, *145*(3), 535-537. https://doi.org/10.1016/j.ijcard.2010.04.067

Ofek, O., Karsak, M., Leclerc, N., Fogel, M., Frenkel, B., Wright, K., ... & Mechoulam, R. (2006). Peripheral cannabinoid receptor, CB2, regulates bone mass. *Proceedings of the National Academy of Sciences*, *103*(3), 696-701. https://doi.org/10.1073/pnas.0504187103

Pazos, M. R., Mohammed, N., Lafuente, H., Santos, M., Martínez-Pinilla, E., Moreno, E., ... & Hillard, C. J. (2013). Mechanisms of cannabidiol neuroprotection in hypoxic–ischemic newborn pigs: Role of 5HT1A and CB2 receptors. *Neuropharmacology*, *71*, 282-291. https://doi.org/10.1016/j.neuropharm.2013.03.027

Ross, R. A. (2009). The enigmatic pharmacology of GPR55. *Trends in pharmacological sciences*, *30*(3), 156-163. https://doi.org/10.1016/j.tips.2008.12.004

Ross, H. R., Napier, I., & Connor, M. (2008). Inhibition of recombinant human T-type calcium channels by Δ9-tetrahydrocannabinol and cannabidiol. *Journal of Biological Chemistry*, *283*(23), 16124-16134. https://doi.org/10.1074/jbc.m707104200

Roy, J., Kazi, M., Hedin, U., & Thyberg, J. (2001). Phenotypic modulation of arterial smooth muscle cells is associated with prolonged activation of ERK1/2. *Differentiation*, *67*(1-2), 50-58. https://doi.org/10.1046/j.1432-0436.2001.067001050.x

Salasznyk, R. M., Klees, R. F., Hughlock, M. K., & Plopper, G. E. (2004). ERK signaling pathways regulate the osteogenic differentiation of human mesenchymal stem cells on collagen I and vitronectin. *Cell communication & adhesion*, *11*(5-6), 137-153. https://doi.org/10.1080/15419060500242836

Steffens, S., Veillard, N. R., Arnaud, C., Pelli, G., Burger, F., Staub, C., ... & Mach, F. (2005). Low dose oral cannabinoid therapy reduces progression of atherosclerosis in mice. *Nature*, *434*(7034), 782-786. https://doi.org/10.1038/nature03389

Taylor, P. M., Batten, P., Brand, N. J., Thomas, P. S., & Yacoub, M. H. (2003). The cardiac valve interstitial cell. *The international journal of biochemistry & cell biology*, *35*(2), 113-118. https://doi.org/10.1016/s1357-2725(02)00100-0

Thomas, A., Baillie, G. L., Phillips, A. M., Razdan, R. K., Ross, R. A., & Pertwee, R. G. (2007). Cannabidiol displays unexpectedly high potency as an antagonist of CB1 and CB2 receptor agonists in vitro. *British journal of pharmacology*, *150*(5), 613-623. https://doi.org/10.1038/sj.bjp.0707133

Qin, N., Neeper, M. P., Liu, Y., Hutchinson, T. L., Lubin, M. L., & Flores, C. M. (2008). TRPV2 is activated by cannabidiol and mediates CGRP release in cultured rat dorsal root ganglion neurons. *Journal of Neuroscience*, *28*(24), 6231-6238. https://doi.org/10.1523/jneurosci.0504-08.2008

Ivanov, V. N., Wu, J., & Hei, T. K. (2017). Regulation of human glioblastoma cell death by combined treatment of cannabidiol, γ-radiation and small molecule inhibitors of cell signaling pathways. *Oncotarget*, *8*(43), 74068. https://doi.org/10.18632/oncotarget.18240

Wagner, E. F., & Nebreda, Á. R. (2009). Signal integration by JNK and p38 MAPK pathways in cancer development. *Nature Reviews Cancer*, *9*(8), 537. https://doi.org/10.1038/nrc2694

Whyte, L. S., Ryberg, E., Sims, N. A., Ridge, S. A., Mackie, K., Greasley, P. J., ... & Rogers, M. J. (2009). The putative cannabinoid receptor GPR55 affects osteoclast function in vitro and bone mass in vivo. *Proceedings of the National Academy of Sciences*, *106*(38), 16511-16516. https://doi.org/10.1073/pnas.0902743106

Appendix: All VIC calcification reduction data.

Concentration	Sample ID	Bioactivity .20	.30	.50	.60	.70	.80	.90	.95
5mg	1548	4.2	5.5	10.4	8.7	8.6	13.0	18.5	21.9
5mg	1730	4.8	5.7	8.2	8.2	13.6	13.8	19.3	19.2
5mg	1194	4.7	5.9	9.1	7.4	11.6	16.4	17.8	21.4
5mg	1785	5.5	6.4	7.8	8.1	11.1	13.1	21.0	21.2
5mg	1772	6.9	6.9	7.5	7.0	10.7	15.1	18.0	18.9
5mg	1169	3.8	6.1	7.1	9.1	10.5	15.3	17.9	19.7
5mg	1245	4.1	5.6	7.8	7.3	9.9	14.2	16.0	19.8
5mg	1496	4.1	6.4	7.9	8.8	10.3	13.8	14.6	19.4
5mg	1381	6.1	6.7	7.6	5.3	10.7	13.9	18.1	20.5
5mg	1380	4.4	5.5	8.2	6.3	10.7	13.6	18.6	16.7
5mg	1230	6.3	5.3	6.2	7.4	7.3	16.0	17.2	21.7
5mg	1655	5.0	6.1	10.0	7.2	11.2	15.0	17.7	18.8
5mg	1175	5.4	6.2	7.4	7.7	11.6	11.3	17.7	19.8
5mg	1617	6.4	6.3	7.8	7.4	8.0	14.0	18.5	17.6
5mg	1042	6.4	6.3	7.5	7.6	9.5	14.1	19.1	18.5
5mg	1640	5.3	6.0	8.3	6.2	9.1	14.6	16.5	18.8
5mg	1082	4.3	5.3	8.1	7.5	11.0	14.6	19.0	16.5
5mg	1743	5.2	7.3	7.9	8.1	9.7	15.9	19.2	18.5
5mg	1278	5.3	6.5	8.2	8.9	10.1	15.4	17.8	22.1
5mg	1134	4.3	6.4	8.1	7.5	9.7	14.6	18.7	20.4
5mg	1452	5.4	5.2	7.8	7.6	9.5	16.5	17.5	16.6
5mg	1696	5.5	6.4	7.7	7.8	8.8	16.6	19.5	19.7
5mg	1765	6.0	5.8	8.7	7.5	9.6	13.3	18.1	18.7
5mg	1032	5.7	6.3	7.8	6.8	10.5	12.4	17.8	21.1
5mg	1850	4.5	6.6	7.7	7.3	9.5	13.1	18.7	21.9
5mg	2018	4.6	6.5	6.8	8.8	10.3	14.7	17.0	15.4
5mg	1205	5.4	7.0	8.2	8.5	8.5	12.3	17.8	19.6
10mg	1548	3.7	6.6	8.8	12.7	15.0	17.5	23.1	32.5
10mg	1730	4.6	6.4	11.2	11.6	14.4	19.1	20.5	18.5
10mg	1194	4.5	6.5	10.0	11.4	13.5	22.8	20.9	24.2
10mg	1785	5.9	6.5	9.3	11.9	12.8	18.9	26.2	27.9
10mg	1772	4.7	5.3	10.2	10.6	16.0	21.7	24.5	26.8
10mg	1169	4.7	5.3	9.1	12.1	15.7	16.2	29.6	24.0
10mg	1245	4.9	5.6	9.1	9.2	13.6	17.1	23.5	20.8
10mg	1496	5.1	7.1	10.8	14.3	10.2	18.8	28.3	31.0
10mg	1381	4.9	5.9	9.2	12.7	12.4	16.4	22.4	27.9
10mg	1380	3.9	6.6	9.2	11.2	15.5	19.5	24.7	25.4
10mg	1230	5.4	7.0	10.9	11.6	15.1	22.5	23.2	25.1
10mg	1655	5.9	5.9	9.5	10.8	15.6	22.9	28.6	27.0
10mg	1175	4.5	6.7	9.9	11.4	13.3	22.0	25.4	22.6
10mg	1617	6.2	6.1	10.6	11.4	14.4	20.9	23.8	28.1
10mg	1042	4.5	6.6	11.4	11.5	15.3	21.9	21.5	27.1
10mg	1640	4.6	6.8	10.6	11.9	14.0	18.6	26.9	22.3
10mg	1082	5.0	5.2	8.6	10.8	17.2	22.7	23.1	28.7
10mg	1743	3.7	4.9	7.7	13.1	13.5	24.1	24.5	24.3
10mg	1278	5.2	5.3	11.7	14.0	16.9	20.2	24.4	23.5
10mg	1134	5.2	6.7	10.0	11.9	15.0	16.0	24.0	25.7
10mg	1452	5.2	6.2	9.1	11.4	14.5	15.9	24.4	26.3
10mg	1696	4.8	6.5	9.2	10.4	13.5	24.6	29.6	24.8
10mg	1765	5.1	6.1	9.1	12.6	13.9	20.7	25.6	27.2
10mg	1032	5.2	7.5	10.2	12.7	12.6	20.3	26.0	27.4
10mg	1850	5.8	6.2	10.8	12.6	13.4	19.0	27.0	21.3

10mg	2018	5.0	5.9	8.8	12.3	14.1	20.8	24.0	25.6
10mg	1205	5.4	7.1	8.8	10.6	15.4	19.8	30.3	24.1
25mg	1548	5.6	5.5	11.9	15.3	16.3	28.5	31.6	34.9
25mg	1730	5.9	7.1	10.3	13.1	14.8	27.7	34.4	27.7
25mg	1194	6.8	6.9	11.8	14.6	16.3	22.4	31.6	34.0
25mg	1785	5.7	6.6	10.7	12.7	14.7	22.7	28.3	29.2
25mg	1772	6.3	6.7	11.1	13.5	18.4	20.2	31.7	34.4
25mg	1169	5.2	6.1	10.5	14.2	18.6	27.2	31.5	30.3
25mg	1245	6.0	7.2	10.4	13.1	17.0	29.9	25.5	34.8
25mg	1496	5.5	8.1	11.0	13.5	17.0	25.9	30.1	31.6
25mg	1381	5.8	6.1	11.5	13.8	15.0	23.3	35.1	31.4
25mg	1380	6.3	4.9	10.0	14.6	15.5	25.0	29.1	42.4
25mg	1230	5.6	7.6	10.2	13.1	18.4	27.7	33.1	29.7
25mg	1655	7.1	4.9	12.1	13.4	15.8	28.2	30.8	42.6
25mg	1175	6.9	6.8	10.9	14.3	15.6	23.9	29.1	32.3
25mg	1617	5.7	4.6	9.8	12.8	18.0	24.6	28.4	35.6
25mg	1042	6.1	7.0	11.9	10.5	17.8	30.3	23.5	34.1
25mg	1640	5.7	5.0	11.1	14.0	16.3	25.5	32.9	31.0
25mg	1082	6.4	6.6	11.4	13.3	17.9	24.6	27.9	30.4
25mg	1743	6.2	5.6	11.4	14.6	14.3	22.4	26.8	29.7
25mg	1278	5.4	6.9	10.7	13.3	14.6	24.2	33.7	39.3
25mg	1134	6.7	6.4	11.5	15.0	17.9	22.6	27.5	34.3
25mg	1452	6.5	6.9	10.6	14.2	17.6	23.1	27.1	38.9
25mg	1696	4.8	6.2	11.3	14.2	16.9	23.2	35.6	28.6
25mg	1765	6.4	5.6	12.5	14.2	16.6	26.5	29.9	32.2
25mg	1032	6.4	5.4	9.8	13.2	18.3	25.2	30.4	31.9
25mg	1850	6.2	6.3	10.4	13.7	16.7	24.9	31.0	26.5
25mg	2018	6.3	5.3	9.7	14.8	15.0	20.6	33.2	28.6
25mg	1205	6.4	5.9	9.5	14.3	15.6	23.1	33.9	26.3
40mg	1548	3.8	7.9	12.4	21.9	30.0	32.3	40.6	39.6
40mg	1730	5.2	7.8	16.0	18.1	28.2	27.6	34.7	43.0
40mg	1194	5.3	7.6	12.1	20.4	24.6	32.8	42.1	47.9
40mg	1785	4.9	7.8	13.2	20.2	24.7	34.6	44.6	42.6
40mg	1772	5.2	7.5	13.9	17.3	24.8	23.4	37.5	47.7
40mg	1169	4.7	5.8	17.1	18.0	29.7	30.6	39.6	46.8
40mg	1245	4.6	6.6	14.9	19.8	25.7	29.7	38.1	47.1
40mg	1496	4.7	8.0	14.9	21.2	19.9	29.1	39.4	44.8
40mg	1381	4.4	6.0	13.2	17.6	22.1	29.0	38.5	46.7
40mg	1380	6.3	7.7	14.7	16.8	24.3	27.9	42.4	36.5
40mg	1230	5.4	6.9	16.5	19.2	33.0	29.4	41.5	36.7
40mg	1655	5.5	6.2	15.4	18.6	22.6	29.2	43.1	43.0
40mg	1175	3.6	7.3	13.8	20.4	24.9	26.7	36.2	38.5
40mg	1617	4.5	6.9	13.3	19.3	18.9	22.4	42.3	51.1
40mg	1042	5.0	6.7	12.6	21.7	25.9	33.9	43.1	41.6
40mg	1640	4.7	8.1	13.2	17.1	22.5	26.2	42.1	46.1
40mg	1082	5.3	7.7	15.0	19.7	22.8	29.5	38.8	37.9
40mg	1743	6.2	6.8	13.9	20.2	24.3	25.3	36.0	36.9
40mg	1278	5.9	8.1	15.2	17.6	19.8	23.6	40.2	44.1
40mg	1134	5.7	7.0	14.2	21.6	24.4	27.0	39.4	42.1
40mg	1452	5.2	7.2	14.2	19.7	23.9	26.5	40.1	39.2
40mg	1696	6.0	6.8	12.0	17.9	25.3	27.6	39.6	35.8
40mg	1765	6.9	8.0	14.8	17.4	23.2	30.6	41.9	39.6
40mg	1032	5.2	6.6	15.2	20.6	25.5	31.6	33.7	38.9
40mg	1850	5.0	5.1	16.3	18.8	27.4	27.9	42.8	44.1
40mg	2018	6.1	7.0	12.6	18.5	21.4	27.8	43.4	47.6
40mg	1205	4.7	7.3	12.7	19.6	27.2	28.4	40.7	54.5

100mg	1548	6.0	7.9	16.1	23.7	30.2	41.6	51.8	52.5
100mg	1730	6.5	6.9	16.0	19.9	26.8	38.8	51.9	43.0
100mg	1194	5.3	8.4	16.5	21.6	35.6	40.1	49.3	59.8
100mg	1785	5.8	6.9	14.9	21.7	31.6	35.8	47.4	55.6
100mg	1772	6.1	7.4	17.9	20.7	30.5	39.2	53.8	59.0
100mg	1169	5.4	7.3	14.8	21.7	29.7	44.4	47.6	50.7
100mg	1245	6.1	8.1	15.3	22.6	32.2	39.2	49.8	51.4
100mg	1496	5.8	6.7	14.1	21.4	29.4	33.8	53.6	41.9
100mg	1381	6.5	7.7	15.7	22.1	29.3	35.6	51.6	60.4
100mg	1380	6.5	6.7	16.4	20.5	30.4	39.6	45.2	62.0
100mg	1230	6.8	7.6	16.4	18.3	29.6	38.2	44.9	66.1
100mg	1655	6.5	6.9	15.0	22.5	31.9	36.3	50.3	59.6
100mg	1175	5.7	6.5	15.9	22.5	31.1	36.1	44.4	63.2
100mg	1617	5.2	7.8	18.1	21.8	31.1	37.1	45.0	51.5
100mg	1042	6.2	6.7	16.5	21.6	27.7	35.0	53.9	57.0
100mg	1640	6.6	6.0	17.1	20.5	30.4	39.7	38.3	64.7
100mg	1082	5.5	6.5	15.5	21.3	31.5	31.7	54.7	58.4
100mg	1743	6.4	7.1	16.7	20.7	29.6	41.8	46.6	56.7
100mg	1278	7.4	7.4	15.8	21.8	32.0	39.5	37.6	41.9
100mg	1134	5.9	7.5	14.3	19.3	31.5	39.7	46.0	51.9
100mg	1452	5.8	7.3	17.3	22.8	33.1	37.2	51.5	43.6
100mg	1696	7.5	8.4	15.7	22.0	35.0	38.5	51.8	56.6
100mg	1765	5.8	7.6	15.4	22.4	32.2	35.9	47.4	56.3
100mg	1032	6.5	6.2	17.3	22.4	27.9	36.5	47.9	59.3
100mg	1850	5.7	6.7	17.4	23.3	31.5	39.9	47.5	52.0
100mg	2018	5.6	6.6	15.5	20.3	31.7	34.8	50.3	53.6
100mg	1205	5.6	7.6	17.2	22.3	25.8	33.1	50.0	56.4

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Bioactive cannabidiol more greatly reduces valvular interstitial cell calcification when combined with ß-Caryophyllene, and α-Humulene

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Abstract:

Valvular interstitial cells (VICs) are integral to heart valve homeostasis and structural leaflet integrity. Aberrant calcification of VICs leads to dangerous diseases including calcific aortic valve disease. VIC calcification can be reduced by cannabidiol (CBD) through modulation of the ERK cascade by selective antagonism of the CB2 receptor, and possible involvement of the GPR55 receptor. ß-Caryophyllene (BCP) and α-Humulene (HMU) are sequiterpenes that produce their own effects on calcification. The present study aimed to see whether a combination of CBD, BCP, and HMU (ImmunAG) could reduce calcification to a greater extent than CBD alone. VICs were isolated from porcine aortic valve leaflets, induced to calcify, and treated with CBD or ImmunAG. Treatment concentrations of 5, 10, 25, 40, and 100 mg were examined. Means, standard deviations, minimum, and maximum calcification reduction values for each treatment and mg concentration are provided. 5 *t*-tests revealed that ImmunAG reduced calcification more than CBD at every concentration.

Within the complex multichambered pump that is the human heart exist supple, flow-regulating membranes known as heart valves (Mohler et al., 2001; Butcher, Simmons, & Warnock, 2008). Within these valves, among an extracellular matrix, exist valvular interstitial cells (VICs), which are integral to valve homeostasis and structural leaflet integrity (Hjortnaes et al., 2015; Taylor, Batten, Brand, Thomas, & Yacoub, 2003).

Calcium salts can be abnormally deposited among soft tissues, causing them to harden and reducing their capacity to function properly. Calcification of VICs causes disruption of interstitial cell mechanical phenotype, and drives interstitial matrix disorganization, nodule formation, and pro-calcific signaling (Farrar, Pramil, Richards, Mosher, & Butcher, 2016). This can lead to any number of dystrophic calcification diseases of the heart that each can increase the risk for developing other cardiovascular diseases (e.g., valvular stenosis, see Michel & Dipchand, 2017; de Simone et al., 2010; Gerdts et al., 2015).

A previous study by Cushing, Goakar, and Joseph (2018), showed that cannabidiol (CBD) extracted from Kriya Brand Humulus significantly reduced VIC calcification. Higher bioactivity CBD caused greater reductions. The highest bioactivity CBD (bioactivity = .95) at the highest concentration (100mg) caused a mean reduction of 55% (*SD* = 6.66). While this is certainly noteworthy, the addition of other synergistic phyo-compounds may reduce calcification further.

β-Caryophyllene (BCP) is a natural bicyclic sesquiterpene abundantly found in essential oils from various spices, fruits and plants. It is approved by United States Food and Drug Administration and European agencies as food additive, taste enhancer and flavoring agent and considered a dietary phytocannabinoid (Sharma, M Al Kaabi, Nurulain, Goyal, Amjad Kamal, & Ojha, 2016). BCP has anti-inflammatory, antibiotic, antioxidant, anticarcinogenic and local anaesthetic properties (Legault & Pichette, 2007). It is a functional selective agonist for the CB_2 receptor (Sharma et al., 2016; Bahi, Al Mansouri, Al Memari, Al Ameri, Nurulain, & Ojha, 2014; Gertsch et al., 2008), which attenuates ERK activity (Gu & Masters, 2009), and the peroxisome-proliferator activating receptor alpha (PPARα; Poddighe et al., 2018), which exerts anti-inflammatory effects in the vascular wall (Zandbergen & Plutzky, 2007).

α-Humulene (HMU) is an isomer of BCP. HMU is generally not studied in isolation. It has no affinity for CB_2. It exerts anti-calcific effects indirectly, through reduction of inflammation. It can prevent the production of pro-inflammatory cytokines by suppressing NF-kB activation in macrophages (Yoon et al., 2010).

BCP and HMU can be extracted from Kriya Brand Humulus without adversely affecting the bioactivity of the CBD. The present article examines the effects that a proprietary blend of high bioactivity CBD, BCP, and HMU, called ImmunAG, has on VIC calcification compared to high bioactivity CBD alone.

Results:

Multiplate wells containing calcifying VICs from 27 different tissue samples were treated with 5, 10, 25, 40, or 100mg of either CBD or ImmunAG. Average calcification was computed using the number of nodules per well, and the average area per nodule. Mean nodule area for untreated wells was 3.27 mm^2 (*SD* = 0.32, *min* = 0.5, *max* = 4.0) per well. All reported percent reductions in calcification were computed by dividing average nodule area of treated wells from 3.27 mm^2.

Means, standard deviations, and minimum and maximum values for CBD and ImmunAG for every tested mg concentration are provided in Table 1. (Full data are provided in the Appendix.) Paired-samples *t*-tests determined that ImmunAG reduced calcification significantly more than CBD alone at every mg concentration: At 5mg, $t(26) = 9.87$, $p < .001$, $d = 1.90$; at 10 mg, $t(26) = 4.20$, $p < .001$, $d = 0.81$; at 25 mg, $t(26) = 5.23$, $p < .001$, $d = 1.00$; at 40 mg, $t(26) = 7.76$, $p < .001$, d

mg		CBD	ImmunAG
5	*M*	19.42	24.32
	SD	1.8	1.77
	Min	15.4	20.1
	Max	22.1	27.8
10	*M*	25.56	30.39
	SD	3.04	4.97
	Min	18.5	19
	Max	32.5	39.7
25	*M*	32.69	38.53
	SD	4.33	4.44
	Min	26.3	26.9
	Max	42.6	46.1
40	*M*	42.98	52.83
	SD	4.8	3.65
	Min	35.8	44.6
	Max	54.5	60
100	*M*	55	64.21
	SD	6.66	7.91
	Min	41.9	51.3
	Max	66.1	78.1

Table 1: Descriptive statisticis of calcification reduction data of CBD and ImmunAG by mg concentration,

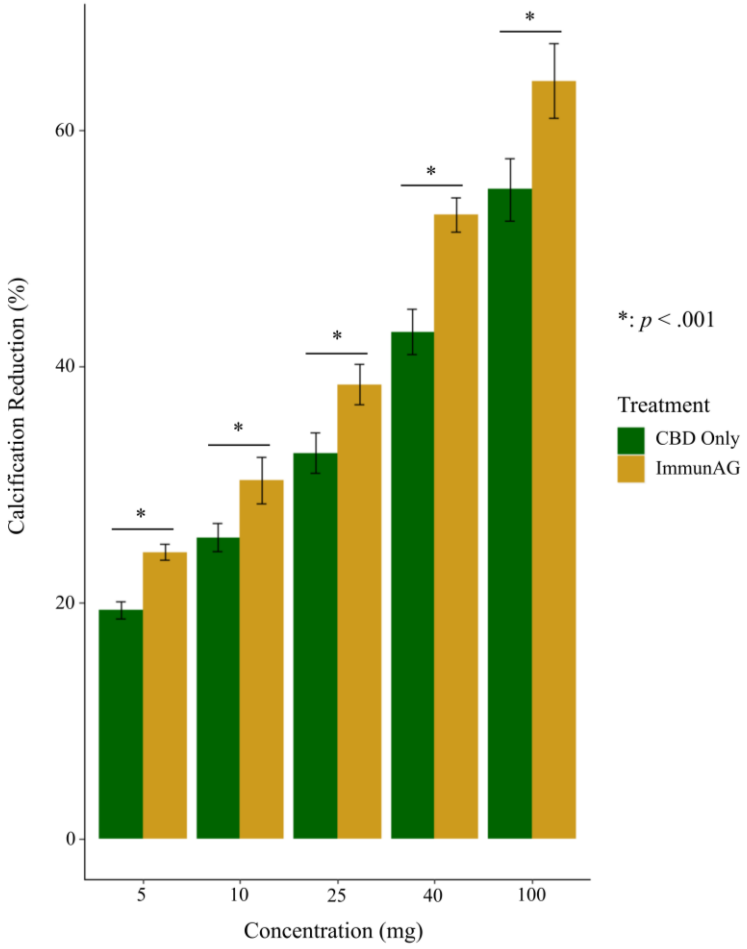

Figure 1: ImmunAG reduced calcification significantly more than CBD at every mg concentration.

= 1.49; and at 100 mg, $t(26) = 4.05$, $p < .001$, $d = 0.78$. A barplot of observed differences is given in Figure 1.

Conclusion:

This study demonstrated that a combination of high bioactivity CBD, BCP, and HMU reduced VIC calcification more than high bioactivity CBD alone. It remains to be seen whether possible synergistic effects afforded by this combination of compounds extends beyond VIC calcification. CBD has been shown to have anxiolytic, antidepressant, antipsychotic, anticonvulsant, anti-nausea, antioxidant, antiinflammatory, anti-arthritic, and anti-neoplastic properties (Ligresti Petrocellis, & Di Marzo, 2016). BCP has shown promising for anti-endemic, anti-tumoral, anti-oxidant, anti-microbial, and anti-inflammatory properties (Dahham et al., 2015). As phytoceutical approaches to medicine continue to gain traction, a uncovering the ways that each of these properties interact will constitute an exciting new frontier for science.

Method:

This study was carried out concurrently with the study described in Cushing, Goakar, & Joseph (2018). All chemicals and solutions were obtained from Sigma-Aldrich (St. Louis, MO). All cell cultures were obtained from Creative Bioarray, Shirley, NY. CBD bioactivity was measured using practices described in Cushing, Kristipati, Shastri, and Joseph (2018).

VIC isolation and culture

VICs were isolated from porcine aortic valve leaflets (Hormel, Austin, MN) by collagenase digestion and subsequently cultured in growth medium (15% FBS, 2 mML-glutamine, 100 U/ml penicillin, and 100 g/ml streptomycin in medium 199) at 37°C, 5% CO2 for two to four passages. VICs used in all experiments were seeded at a density of 50,000 cells/cm^2 onto 24-well or 96-well plates. During the experiments, the VICs were cultured in low-serum medium (1% FBS, 100 U/ml penicillin, 100 g/ml streptomycin, 2 mM L-glutamine, in medium 199), and the medium was changed each day until the fifth day.

Culture substrate coatings

Tissue culture polystyrene (TCPS) plates (24-well or 96-well) were coated with type I collagen (Coll) (Inamed Biomaterials, Fremont, CA; 2 g/cm2), fibronectin (FN, 5 g/cm2), fibrin (FB, 1.5 g/cm2), or left untreated (TCPS). For the FB coating, plates were first incubated overnight at 4°C in fibrinogen (1 mg/ml), followed by three washes with 0.05% Tween 20 in phosphate- buffered saline (PBS) and 1 h incubation with thrombin (0.6 mg/ml) at 37°C (12). All coatings were prepared in 50 mM bicarbonate coating buffer, pH 8.5, and rinsed three times with PBS before cell seeding. The amounts of adsorbed proteins were measured on separate plates using the bicinchoninic acid protein assay (Pierce, Rockford, IL) to verify adsorption of protein coatings.

MEK-1/2 inhibition

VICs exposed to various concentrations and bioactivities of CBD were treated with U-0126 [1,4-diamino- 2,3-dicyano-1,4-bis(2- aminophenylthio) butadiene; Calbiochem, San Diego, CA], PD-98059 (2-amino-3 methoxyflavone; 5 M; Calbiochem), or left untreated as a control to confirm the MAPK specificity of these inhibition experiments. U-0126 specifically inhibits MEK-1/2, thus inhibiting activation of ERK-1/2 (Favata et al., 1998). PD-98059 is an alternate MEK inhibitor (Citation). 9 tissue samples were in each treatment group. These were the tissue samples used in subsequent analyses.

Quantification of cell number

At time points of 1, 3, and 5 days, VICs were lysed with radioimmunoprecipitation assay buffer [1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 1 mM iodoacetamide, 140 mM NaCl, 10 mM Tris HCl, (pH 8.0)]. The amount of DNA in sample lysates was measured via the Quanti-iT PicoGreen assay (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions.

Migration assay

Migration was assayed via a modified fence method (Mann & West, 2002), wherein VICs were seeded within 2 mm^2 removable silicone wells, grown to confluency, and then allowed to migrate following the detachment of silicone isolators (defined as day 0). Grid-patterned transparencies were attached underneath plates containing VIC cultures to track cell movement over time. Photomicrographs were taken of the leading edge of cell migration under 40 magnification (Olympus IX51) every 24 h for 5 days. Net cell edge displacement was measured

by overlaying time course images and then quantifying migration distance (NIH ImageJ) by measuring the advancement of the leading cell edge subtracted from the migration area recorded on day 0 within a single grid space.

Apoptosis assay

To ensure the health of the cell samples used in the calcification experiment, apoptosis was measured using an ELISA-based HT TiterTACS Assay Kit (Trevigen, Gaithersburg, MD), which detects DNA fragmentation. At days 1 and 5, cells were fixed in 3.7% buffered formaldehyde solution for 7 min, washed with PBS, and postfixed in 100% methanol for 20 min. Following manufacturer's instructions, the cells were permeabilized with proteinase K, quenched with 2.5% H2O2 in methanol, and then incubated with the labeling reaction mix (TdT, Biotin-dNTP, unlabeled dNTP) to label breaks in DNA. Streptavidin-HRP and then TACS-Sapphire were added to the wells to detect apoptotic cells; the reaction was stopped with 2 N HCl, and absorbance was read at 450 nm.

RNA isolation

Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH), according to the manufacturer's instructions. VICs were lysed with 200 l TRI Reagent per well at 4°C with 50 protease inhibitor cocktail (BD Biosciences, San Jose, CA). The homogenate was stored at room temperature for 5 min to complete the dissociation of nucleoprotein complexes, at which point 0.15 ml chloroform per 600 l TRI Reagent was added to the homogenate, followed by centrifugation at 13,000 g for 15 min. After centrifugation, RNA was precipitated from the upper aqueous phase by adding 0.3 ml isopropanol per 600 l TRI Reagent to the tubes and then centrifuged at 13,000 g for 8 min. After this centrifugation step, the RNA pellet was washed with 75% ethanol and centrifuged at 8,000 g for 5 min. The RNA pellet was air dried and dissolved in 75l H2O at 60°C for 15 min. RNA samples were stored at 20°C until subsequent use.

Quantitative real-time PCR analysis

Custom primers for various markers of cell contractility and osteogenic activity were obtained from Invitrogen (Carlsbad, CA) and are listed in Table 1. For cDNA construction, 250 ng of original RNA isolated from samples were reverse transcribed using iScript (Bio-Rad Laboratories, Hercules, CA) as per manufacturer's instructions. Samples were processed for real-time PCR analysis by combining 0.5 l of the cDNA construction, 5 M of primers, and SYBR Green SuperMix (Bio-Rad) in a 15-l reaction, as specified in the manufacturer's protocol. For thermo cycling, a standard protocol was used: PCR reactions were run over 40 cycles of denaturing at 95°C for 15 s and annealed at 60°C for 1 min; this was followed by a melting curve analysis for 80 cycles of 55°C 0.5°C/cycle, 10 s per cycle, to further confirm the purity of the final PCR products, with each condition performed in triplicate (iCycler iQ Real-Time PCR Instrument, Bio-Rad). A standard comparative threshold cycle (or CT) method was used to analyze the PCR data. The CT of all samples were first normalized to -actin as an internal control, and then the CT values for experimental samples were further normalized to the negative control (VICs on Coll, which represented a non CBD condition).

Quantification of nodule number and size

After 5 days of culture in the presence or absence of U-0126 or PD-98059, VIC cultures were stained with Alizarin Red S (ARS) to facilitate quantification of calcified nodules, as ARS stains mineralized deposits red. Cultures were fixed with 10% neutral buffered formalin, stored at 4°C overnight, and stained with a 2% solution of ARS in PBS. Positively stained nodules were manually counted under a microscope (Olympus IX51 with Hamamatsu 285 digital camera and Simple PCI digital imaging software; Compix, Imaging Systems, Cranberry Township, PA). Nodule size was measured using ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij/), and photomicrographs were captured under 40 and 100 magnifications.

CBD and ImmunAG samples

.95 bioactivity CBD was isolated by HPLC from ImmunAG, a humulus product of ImmunAG LLP. ImmunAG is a proprietary combination of CBD (39.5%), BCP (59.5%), and HMU(1%). The bioactivity of the BCP and HMU were not directly tested. They were likely approximately equal to the bioactivity of the CBD.

References:

Bahi, A., Al Mansouri, S., Al Memari, E., Al Ameri, M., Nurulain, S. M., & Ojha, S. (2014). β-Caryophyllene, a CB2 receptor agonist produces multiple behavioral changes relevant to anxiety and depression in mice. *Physiology & behavior*, *135*, 119-124. https://doi.org/10.1016/j.physbeh.2014.06.003

Butcher, J. T., Simmons, C. A., & Warnock, J. N. (2008). Mechanobiology of the aortic heart valve. *Journal of Heart Valve Disease*, *17*(1), 62. https://doi.org/10.1007/978-1-4939-5617-3_12

Cheng, Y., Dong, Z., & Liu, S. (2014). β-Caryophyllene ameliorates the Alzheimer-like phenotype in APP/PS1 Mice through CB2 receptor activation and the PPARγ pathway. *Pharmacology*, *94*(1-2), 1-12. https://doi.org/10.1159/000362689

Cushing, D., Goakar, D., Joseph, B. (2018). Differential calcification reduction of valvular interstitial cells by cannabidiol bioactivity and concentration. *Journal of Medical Phyto Research*, *2*(2), 14-22. https://doi.org/10.31013/2001f

Cushing, D., Kristipati, S., Shastri, R., & Joseph, B. (2018). Measuring the bioactivity of phytocannabinoid cannabidiol from cannabis sources, and a novel non-cannabis source. *Journal of Medical Phyto Research*, *10*.

de Simone, G., Izzo, R., Chinali, M., De Marco, M., Casalnuovo, G., Rozza, F., ... & De Luca, N. (2010). Does information on systolic and diastolic function improve prediction of a cardiovascular event by left ventricular hypertrophy in arterial hypertension?. *Hypertension*, *56*(1), 99-104. https://doi.org/10.1161/hypertensionaha.110.150128

Farrar, E. J., Pramil, V., Richards, J. M., Mosher, C. Z., & Butcher, J. T. (2016). Valve interstitial cell tensional homeostasis directs calcification and extracellular matrix remodeling processes via RhoA signaling. *Biomaterials*, *105*, 25-37. https://doi.org/10.1016/j.biomaterials.2016.07.034

Favata, M. F., Horiuchi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feeser, W. S., ... & Copeland, R. A. (1998). Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *Journal of Biological Chemistry*, *273*(29), 18623-18632. https://doi.org/10.1074/jbc.273.29.18623

Gerdts, E., Rossebø, A. B., Pedersen, T. R., Cioffi, G., Lønnebakken, M. T., Cramariuc, D., ... & Devereux, R. B. (2015). Relation of left ventricular mass to prognosis in initially asymptomatic mild to moderate aortic valve stenosis. *Circulation: Cardiovascular Imaging*, *8*(11), e003644. https://doi.org/10.1161/circimaging.115.003644

Gertsch, J., Leonti, M., Raduner, S., Racz, I., Chen, J. Z., Xie, X. Q., ... & Zimmer, A. (2008). Beta-caryophyllene is a dietary cannabinoid. *Proceedings of the National Academy of Sciences*, *105*(26), 9099-9104. https://doi.org/10.1211/jpp.59.12.0005

Gu, X., & Masters, K. S. (2009). Role of the MAPK/ERK pathway in valvular interstitial cell calcification. *American Journal of Physiology-Heart and Circulatory Physiology*, *296*(6), H1748-H1757. https://doi.org/10.1152/ajpheart.00099.2009

Hjortnaes, J., Shapero, K., Goettsch, C., Hutcheson, J. D., Keegan, J., Kluin, J., ... & Aikawa, E. (2015). Valvular interstitial cells suppress calcification of valvular endothelial cells. *Atherosclerosis*, *242*(1), 251-260. https://doi.org/10.1016/j.atherosclerosis.2015.07.008

Legault, J., & Pichette, A. (2007). Potentiating effect of β-caryophyllene on anticancer activity of α-humulene, isocaryophyllene and paclitaxel. *Journal of Pharmacy and Pharmacology*, *59*(12), 1643-1647. https://doi.org/10.1211/jpp.59.12.0005

Ligresti, A., De Petrocellis, L., & Di Marzo, V. (2016). From phytocannabinoids to cannabinoid receptors and endocannabinoids: pleiotropic physiological and pathological roles through complex pharmacology. Physiological reviews, 96(4), 1593- 1659. https://doi.org/10.1152/physrev.00002.2016

Mann, B. K., & West, J. L. (2002). Cell adhesion peptides alter smooth muscle cell adhesion, proliferation, migration, and matrix protein synthesis on modified surfaces and in polymer scaffolds. *Journal of biomedical materials research*, *60*(1), 86-93. https://doi.org/10.1002/jbm.10042

Michel, E. J. S., & Dipchand, A. I. (2017). Valvular Stenosis and Heart Failure. *Heart Failure in the Child and Young Adult: From Bench to Bedside*, 307. https://doi.org/10.1016/b978-0-12-802393-8.00023-5

Mohler III, E. R., Gannon, F., Reynolds, C., Zimmerman, R., Keane, M. G., & Kaplan, F. S. (2001). Bone formation and inflammation in cardiac valves. *Circulation*, *103*(11), 1522-1528. https://doi.org/10.1161/01.cir.103.11.1522

Poddighe, L., Carta, G., Serra, M. P., Melis, T., Boi, M., Lisai, S., ... & Quartu, M. (2018). Acute administration of beta-caryophyllene prevents endocannabinoid system activation during transient common carotid artery occlusion and reperfusion. *Lipids in health and disease*, *17*(1), 23. https://doi.org/10.1186/s12944-018-0661-4

Sharma, C., M Al Kaabi, J., M Nurulain, S., N Goyal, S., Amjad Kamal, M., & Ojha, S. (2016). Polypharmacological properties and therapeutic potential of β-caryophyllene: a dietary phytocannabinoid of pharmaceutical promise. *Current pharmaceutical design*, *22*(21), 3237-3264. https://doi.org/10.2174/1381612822666160311115226

Taylor, P. M., Batten, P., Brand, N. J., Thomas, P. S., & Yacoub, M. H. (2003). The cardiac valve interstitial cell. *The international journal of biochemistry & cell biology*, *35*(2), 113-118. https://doi.org/10.1016/s1357-2725(02)00100-0

Yoon, W. J., Moon, J. Y., Song, G., Lee, Y. K., Han, M. S., Lee, J. S., ... & Hyun, C. G. (2010). Artemisia fukudo essential oil attenuates LPS-induced inflammation by suppressing NF-κB and MAPK activation in RAW 264.7 macrophages. *Food and Chemical Toxicology*, *48*(5), 1222-1229. https://doi.org/10.1016/j.fct.2010.02.014

Zandbergen, F., & Plutzky, J. (2007). PPARα in atherosclerosis and inflammation. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, *1771*(8), 972-982. https://doi.org/10.1016/j.bbalip.2007.04.021

Appendix:

	Treatment	5mg	10mg	25mg	40mg	100mg
1548	CBD	21.9	32.5	34.9	39.6	52.5
1730	CBD	19.2	18.5	27.7	43	43
1194	CBD	21.4	24.2	34	47.9	59.8
1785	CBD	21.2	27.9	29.2	42.6	55.6
1772	CBD	18.9	26.8	34.4	47.7	59
1169	CBD	19.7	24	30.3	46.8	50.7
1245	CBD	19.8	20.8	34.8	47.1	51.4
1496	CBD	19.4	31	31.6	44.8	41.9
1381	CBD	20.5	27.9	31.4	46.7	60.4
1380	CBD	16.7	25.4	42.4	36.5	62
1230	CBD	21.7	25.1	29.7	36.7	66.1
1655	CBD	18.8	27	42.6	43	59.6
1175	CBD	19.8	22.6	32.3	38.5	63.2
1617	CBD	17.6	28.1	35.6	51.1	51.5
1042	CBD	18.5	27.1	34.1	41.6	57

1640	CBD	18.8	22.3	31	46.1	64.7
1082	CBD	16.5	28.7	30.4	37.9	58.4
1743	CBD	18.5	24.3	29.7	36.9	56.7
1278	CBD	22.1	23.5	39.3	44.1	41.9
1134	CBD	20.4	25.7	34.3	42.1	51.9
1452	CBD	16.6	26.3	38.9	39.2	43.6
1696	CBD	19.7	24.8	28.6	35.8	56.6
1765	CBD	18.7	27.2	32.2	39.6	56.3
1032	CBD	21.1	27.4	31.9	38.9	59.3
1850	CBD	21.9	21.3	26.5	44.1	52
2018	CBD	15.4	25.6	28.6	47.6	53.6
1205	CBD	19.6	24.1	26.3	54.5	56.4
1548	ImmunAG	23.7	23.4	41.9	46.8	56.1
1730	ImmunAG	21.3	23	26.9	54.8	74.5
1194	ImmunAG	24.4	26.3	39.2	51.6	78.1
1785	ImmunAG	23.3	30.6	37.6	54.3	73.4
1772	ImmunAG	23.8	39.7	38.4	49.8	53.4
1169	ImmunAG	25	36.1	38.1	49.9	53.7
1245	ImmunAG	25.3	29.2	38.9	52.9	59.1
1496	ImmunAG	21.3	29.2	46.1	57	66.8
1381	ImmunAG	24.2	23.5	38.1	55.7	54.9
1380	ImmunAG	23.4	31.5	35.1	56.6	51.3
1230	ImmunAG	26.5	32.3	45.9	55.3	66.6
1655	ImmunAG	25.4	32	41.4	50.2	57.3
1175	ImmunAG	23.7	34.7	39.6	53.3	56.7
1617	ImmunAG	24.6	31.2	35.7	51.8	56.1
1042	ImmunAG	24.2	19	42.6	53	63.5
1640	ImmunAG	22.9	25.1	42.5	53.1	72.4
1082	ImmunAG	27.5	31.3	36.5	52	70
1743	ImmunAG	24.7	27.8	42.7	50.7	58.8
1278	ImmunAG	25.1	33.5	31.9	49.7	74.5
1134	ImmunAG	27.8	37.7	39.1	57.2	64.3
1452	ImmunAG	25.3	34.1	43.3	60	77.7
1696	ImmunAG	20.1	35.4	38.3	54.1	64.6
1765	ImmunAG	25.1	33	39.6	59.9	71.6
1032	ImmunAG	22.2	27.2	34.3	48	62.8
1850	ImmunAG	26.2	30	29.5	44.6	63.3
2018	ImmunAG	24.7	27.5	37.3	51.7	67.7
1205	ImmunAG	24.9	36.1	39.7	52.4	64.5

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